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THE BACTERIAL CANKER OF POPLARS

BY

HENRIËTTE C. KONING

INTRODUCTION

In different parts of Europe a disease is prevalent among the poplars, a so-called canker, which may prove a great danger to the culture of certain varieties. Especially in connection with the demand for poplarwood, with qualities suitable for industrial purposes, it will be of importance to know, whether the new species and varieties, obtained by selection and crossing, are sensitive to canker. We know of several instances, where varieties, grown in large numbers on account of their valuable qualities, after some years proved to be highly susceptible to this disease.

In the Netherlands for instance, in the years 1860—1870 a certain local variety, *Populus brabantica* Houtzagers (The „Heeswijksche Witte") was grown in large numbers. We may assume on good grounds that this „species" originated in some part of the Netherlands between 1850 and 1860 as a hybrid between *Populus marilandica* Bosc. ♀. and *Populus serotina* Hart ♂. [1]) It excelled by its rapid, upright growth and its fine, white bark, and consequently was in great demand. In 1870 *P. brabantica,* of which many 10 years-old specimen then existed, began to suffer from canker, in 1875 already, hardly any healthy specimen of these trees was to be found (Pl. I fig. 1). Other poplars, among which also *P. serotina* and *P. marilandica* remained altogether free from canker.

Also in Belgium and in Western Germany *P. brabantica* is found and is also susceptible to canker there. In the Netherlands dunes *Populus candicans* Ait. has been planted in great numbers. This

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[1]) In this article the nomenclature of Dr. Houtzagers, as found in „Het geslacht Populus" (Wageningen 1937), will always be used. The trees, which served for his work, are still all to be found in the experimental nurseries of the Ned. Heide Maatschappij and were also used by the author for her experiments.

poplar, which remains low, would be very suitable indeed for the planting of the dunes, but for its great susceptibility to canker. All the dune-bushes die of canker and this process is even accelerated by the frequent occurence of *Saperda carcharias* L. and other wood-boring insects.

Among the local varieties in Belgium and France, known as „régénérés" several are found which are strongly liable to canker, so that whole areas exist, where nearly all the poplars have been attacked. *Populus regenerata* Henry, grown here too, often shows different cankerwounds on the branches; however in this tree, the disease does not seem to spread so easily. In England some species are used, which are highly susceptible; *Populus Eugenei* Simon Louis and *P. candicans Ait.* are mentioned there as being very liable to canker.

About the cause of poplar-canker many contradictory reports can be found in literature. This induced Prof. Joh. Westerdijk to have further investigations carried out. Very soon it appeared that there are various forms of canker, caused respectively by a bacterium and by some species of fungi. By far the most important form in the Netherlands proved to be caused by a bacterium, not described as yet. This form of canker is treated in the present article, whilst by the side of it reference is also made to other forms of canker.

The investigations were carried out in the Phytopathological Laboratory „Willie Commelin Scholten" at Baarn under the supervision of Professor Westerdijk.

Very soon already the „Nederlandsche Heide Maatschappij" offered her assistance and also helped finantially. A nursery was put at the disposal of the investigators, planted with all sorts of poplars, suitable for being investigated as to the susceptibility of the different types to canker. In the periodical of the „Nederlandsche Heide Maatschappij" we have written about experiments, concerning the susceptibility and resistance of these poplars to *Nectria* canker.

In this article nothing can be said as yet about the resistance of the poplar-species and varieties to bacterial canker, as not sufficient time has elapsed as yet, since the beginning of these experiments. The research work will be continued however.

The „Provinciale Waterleiding Maatschappij van Noord-Holland" has also shown itselve interested in the resistance and susceptibility of those species of poplars, suitable for the planting of the dunes. Here too some experimental fields have been laid out, planted with a great assortment of poplar-species, which will all be investigated with regard to their resistance to bacterial canker also.

CHAPTER I

SURVEY OF LITERATURE

In different countries investigations about poplar canker have already been made.

In the *Netherlands* Westerdijk and van Luyk (1924) investigated the fungi — *Nectria coccinea* strains —, which they had isolated from poplar cankers. The investigations which were chiefly mycological, aimed at determining the differentiations between *N. coccinea* (Pers.) Fr. and *N. galligena* Bres. Inoculation experiments were made in order to study the pathogenity of *Nectria coccinea*, which Weese (1911, 1912, 1920) had denied. It was compared with the canker-producing capacity of *N. galligena*. In consequence of the inoculations, which were carried out in autumn and in winter, they obtained small cankers resembling beech-canker more than apple-canker. The experiments however were not continued for a sufficiently long period to be able to follow the further development of the cankers.

In *Germany* the first investigator dealing with poplar canker was Lapine (1892). He inoculated a *Nectria* (which he determined as *N. ditissima* Tul.) in all sorts of trees. In fruit trees and in *Quercus* cankers were formed. In *Populus* all the wounds healed rapidly. This may be due to the fact that the inoculations were carried out on June 21 and August 1. As will be seen from page 31 of this article, the inoculations with *Nectria* in poplars during the summer months have no effect at all. Moritz (1930) compared the virulence of 22 *Nectria* strains (both *coccinea* and *galligena*) on various trees. With two of these strains he obtained infection in *Populus*. The wounds healed however during the vegetation period. He does not give the exact time of inoculating.

Richter (1928) also made inoculation experiments with strains of *Nectria coccinea* (Pers.) Fr. and *Nectria galligena* Bres., which he had isolated from various trees. Only in the case of one strain, obtained from cankers of *P. nigra* L. from Norway, he got poplar canker. The inoculation with this strain took place in October. With all other strains, among which also the strain *Nectria coccinea* (Pers.) Fr. var. *sanguinella* (Fr.) Wr. of Westerdyk and van Luyk, was used, no cankers could be formed. This again may be due to the fact, that the inoculations with them were carried out in July. The other German investigators: Nieszen (1907), Weese (1911), Wollenweber (1913, 1926, 1928, 1935) and Liese (1931) recorded *Nectria ditissima, N. galligena* or *N. coccinea* on poplar canker. They made no inoculations. Good figures are given by Liese.

In *Belgium* the poplar canker was investigated by Nypels in 1897. This author also gives a very good drawing of canker on the stem.

Since I have seen the figures mentioned, I suppose that Nypels like Liese were dealing with the bacterial canker as described in this article.

In his isolations Nypels constantly found *Hyalopus populi* Nypels. Poskin (1903) isolated *N. ditissima* Tul. In march 1902 he carried out inoculations with ascospores of this fungus, taken directly from poplar, apple and beech. In the first two cases open canker wounds were to be seen in June 1903. Delevoy and Boudru (1935) have studied the resistance of various poplar species, planted in different kinds of soil and exposed to spontaneous infection. They isolated *Cylindrocarpon Willkommi* (Lind.) Wr., *Cytospora* spec. and *Macrophoma tumefaciens* Shear. Inoculations with these fungi, separately or combined, resulted in the formation of cankers. As a description of the experiments and the results is lacking, they cannot be compared with the experiments of other investigators. For the rest Delevoy and Boudru are inclined to share the ideas of the English investigators as regards poplar canker.

All this proves that specially in the Netherlands and in Germany but also in Belgium there was every reason to consider *Nectria coccinea* as well as *galligena* to be the chief, if not the

only, cause of poplar canker. The investigations, published in this article, at first also pointed in the same direction; soon however, it appeared, that after the inoculation with *Nectria* no cankers were produced, which were identical with the most frequent type of poplar canker.[1])

In *England* it is thought, that possibly a bacterial canker, as well as a canker caused by fungi, may occur in the poplar. (Day and Peace 1934). The presumption that a bacterium might be the cause of a poplar-canker, is based upon the exudations which were observed to occur so frequently in spring in the cankers of the poplars. *Nectria* fructifications were sometimes found in cankers from which also exudations were flowing. Two types of canker can be distinguished, one, resembling apple-canker and one closed type with fissures; these types however are not supposed to be connected with an organism each separately. The rôle of *Nectria* is left undecided; when speaking of cankers, caused by fungi, a *Dothichiza* infection is generally meant[1]). The description of Day and Peace corresponds in many respects with the observations made by the author.

In *France* Delacroix in 1906 gave a very good description, together with some figures, of the most frequent form of canker of the poplar. He considered a bacterium — *Micrococcus populi* — to be the cause of it. The results of his infection-experiments are very vague; we must assume, that an error has been made here and that *Micrococcus populi* Delacroix is in no way connected with canker, but that it is a saprophyte which happened to be present. As a matter of fact the present French investigators are convinced of that now, and are reinvestigating the disease (Station de Pathologie Végétale Versailles).

In *Italy* Brizi in 1907 observed tumours in poplars, which are ascribed to *Bacillus populi* Brizi. These tumours have often been wrongly referred to in literature as „cankers with a bacterial

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[1]) In Germany, France, England and the U.S.A. mention is often made of a „canker" in the poplar caused by *Dothichiza populea* Sacc. et Briard. All the literature about this will be left out here, as, in our opinion, the *Dothichiza* attack must be considered to belong to the bark anthracnoses and not to the cankers.

origin". Brizi himself however always clearly distinguished these tumours from the ,,French cankers", described bij Delacroix. These investigations were made simultaneously with Smith's discovery of *Pseudomonas tumefaciens* (Smith et Towns.) Stevens. Brizi does not quote Smith, but the latter says in 1911 (Smith 1911 page 18) that the tumours, described by Brizi, must have been caused by *Ps. tumefaciens*. Servazzi (1932) mentions the occurrence of a poplar-canker, which closely resembles the canker-form, treated by Delacroix. The disease is however of little importance in Italy.

In the *U.S.A.* a poplar-canker is described, caused by *Cytospora chrysosperma* (Pers.) Fr. (= *Valsa sordida* Nitschke) (Long 1918, Leach 1919, 1923, Hubert 1920, Hartley and Hahn 1920, Povah 1921, Moss 1922, Brown 1922, Sanford 1931, Schreiner 1931, Dearness and Hansbrough 1934). In the Netherlands this fungus is mostly found on dead poplar-branches. Also from dead branches on which no pycnidia are to be seen, it can nearly always be isolated. Many observations made by the author on branches dying off, either spontaneously, just above a basal canker-wound or above an inoculation executed with bacteria or fungi, proved the presence of *Cytospora*. This fungus however never was the direct cause of the dieback and neither was it always the first saprophyte which showed itself. In the U.S.A. also *Hypoxylon pruinatum* (Klotsch) Cke. is named as a fungus, which causes canker of the poplar (Povah 1922, 1924; Schreiner 1925). In connection with *Macrophoma tumefaciens* Shear branch tumours are described, which closely resemble the tumours of *Pseudomonas tumefaciens* (Smith et Towns.) Stevens, or the galls produced by *Saperda populnea* L. (Hubert, 1915; Hyde, 1922). Regarding *Dothichiza* cankers, which are also described in the U.S.A. we refer again to the note on page 6.

Togashi (1924) has recorded the occurence of *Valsa sordida* Nitschke on the Lombardy poplars near Hokkaido. *Japan*.

We cannot give our opinion about the literature on frost canker as we did not yet have the opportunity to make observations about this injury ourselves.

For the sake of completeness we had also better mention ,,ash

canker" in this survey of literature. The morphological characteristics of this canker very much resemble those of the bacterial poplar canker. In 1922 C. O. Smith obtained cankers on ash after inoculation of *Pseudomonas Savastanoi* (Smith) Stevens. He could not reisolate the pathogen however. Brown in 1932 isolated a bacterium from ash-canker. With pure cultures of it she obtained cankers on the ash. This confirmed the presumption of the older investigators, that this canker was of a bacterial nature (Noack, 1893; Smith, 1908, 1920, 1922; van Vliet 1931). The bacteriological investigations of Brown (1932) proved that the bacterium in most of its characteristics agrees with *Pseudomonas Savastanoi* (Smith) Stevens. It does not attack the olive, neither can *Pseudomonas Savastanoi* infect the ash. For that reason Brown considers the bacterium of the ash canker to be a variety and calls it *Bacterium Savastanoi* var. *fraxini*.

A Nectria canker in the ash is also described, which is caused by *Nectria galligena* var. *major* Wr. (Wollenweber, 1913, 1926, 1928; Richter, 1928; van Vliet, 1931).

CHAPTER II

BACTERIAL CANKER OF THE POPLAR

The concept „canker".

Appel and Westerdijk in 1919 clearly outlined the concept „canker" in phytopathology by reducing it to the anthracnose of the bark, as the first stage of a canker always is a sunken spot.

The second stage, the reaction of the plant in the form of callus, makes the anthracnose develop into canker, the essential characteristic of a canker being that the wound will not heal.

In human pathology canker has two forms: wounds that will not heal, so-called open wounds, and tumours. Many investigators in phytopathology have used the word canker for these two forms. Appel and Westerdijk however have restricted the vegetable canker to the open wound form only. The swellings have been called tumours by them. In many handbooks however, the word canker is used in a very extensive sense.

On page 21 I will explain whether the term canker may be applied to the bacterial poplar excrescences or not.

In horticulture the term frost canker is usual. The effect of frost upon woody parts of plants is the formation of a fissure, which in the course of time is overgrown and closed in by callus. It is only when the fissure is infected by a parasite, on account of which the wound will not heal, that we may speak of „canker". It is clear that the term frost-canker is used in a too general sense. Many intermediate stages are possible and it is only by giving a distinct description of the etiology of the „canker", that its true nature will be revealed.

§ 1. *Description of the symptoms of the bacterial canker.*

Bacterial canker may be best and briefest described by comparing it with the *Nectria* canker of the apple, as the latter has been studied most extensively and may be considered the prototype of canker in trees.

1. The initial stage of bacterial canker of the poplar shows a local swelling of the bark in the spring; the diseased parts generally have a somewhat lighter colour than their surroundings.

   In the case of *Nectria* canker of the apple on the other hand we find dark-coloured and sunken areas. The initial stage here is a local lesion or anthracnose of the bark.

2a. In the case of bacterial canker of the poplar we find rents and fissures occurring in the swollen bark, from which in May and June a slimy exudation is flowing.

2b. This slime may also flow directly from the bark without a previous swelling, for instance through the lenticels. Then round white masses of slime occur (fig. 1a, d). The whole tissue of the bark proves to have been infected from inside, so that the lenticels soon become bursts, rents and fissures through the onflowing masses of slime (fig. 1b, d).

   When a microscopic cross section is made, we find a dead tissue in the bark with the cells lying loose, because the middel lamella has been dissolved.

   When the weather is dry, the clots of slime as well as the exudations from the rents, become hard shiny layers (fig. 1b, c). When placing branches covered with such layers into the water, these layers will swell and become masses of slime again (fig. 1d).

   Thin branches will die of the infection and all kinds of fungi will follow the bacterial infection (fig. 1c).

   A corresponding phenomenon is not found in the case of *Nectria* canker of the apple.

3. During the summer small cankers will occur on the thicker branches of the poplar, caused by a callus formation near to the rents and fissures. These small cankers have a very irregular („crumbly”) structure (fig. 2).

Fig. 1. One year old twigs of *Populus brabantica* Houtz, with clots of bacterial slime. *a*. First stage (dry), clots coming out of lenticels. *b*. Older stage with cracks and fissures in the bark. *c*. Final stage with secundary fungi. *d*. Young stage, put in water; the clots have swollen. (Drawn by Dr. J. Y. v. Vliet).

Fig. 2. *Populus candicans* Ait. (Balsam poplar). Canker caused by *Pseudomonas rimaefaciens* n. sp.
(Drawn by Dr. J. Y. v. Vliet).

In the case of the apple canker regular smooth callus walls are found around the wound.

4a. In bacterial canker the bark around the small canker of the first year will swell again in early summer of the second year. New rents will be formed near to which irregular callous walls are formed again later on (fig. 3c).

4b. Sometimes we may see slime-clots protruding from the lenticels near the cankers without any previous swelling of the bark. These clots of slime may in their turn be followed by small cankers or may cause a dieback of the branch.

In the case of *Nectria* canker of the apple the spreading takes place concentrically. In summer, during the active period of the tree, a callous wall will be formed, which in winter is followed again by a concentric zone of attacking on the outside of the wall (compare fig. 8).

5. The spreading of bacterial canker takes place internally along small lines in wood or bark, looking like a „red point" when a cross section is made (fig. 3a, b). The red line may extend for

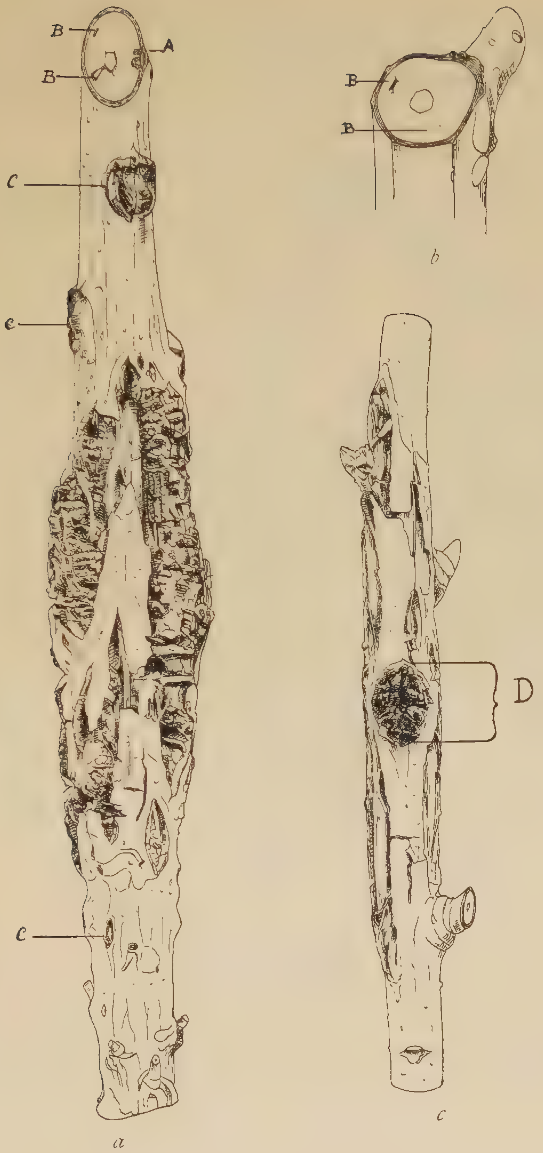


Fig. 3. Spreading of *Pseudomonas rimaefaciens* n. sp. through the branch. *a.* and *b.* (Case 4a): Spreading along the red line B; small secundary cankers C are formed when this line comes in contact with the bark (A). c. (Case 4b).: The next year the initial canker D is enlarged by a general infection of the branch around this canker. D is black, the secundary outgrowth of the canker is brown. (Drawn by Dr. J. Y. v. Vliet).

quite a long distance on branches which externally are perfectly healthy. At a certain place in radial direction, it will come into contact with the bark and cause the latter to swell

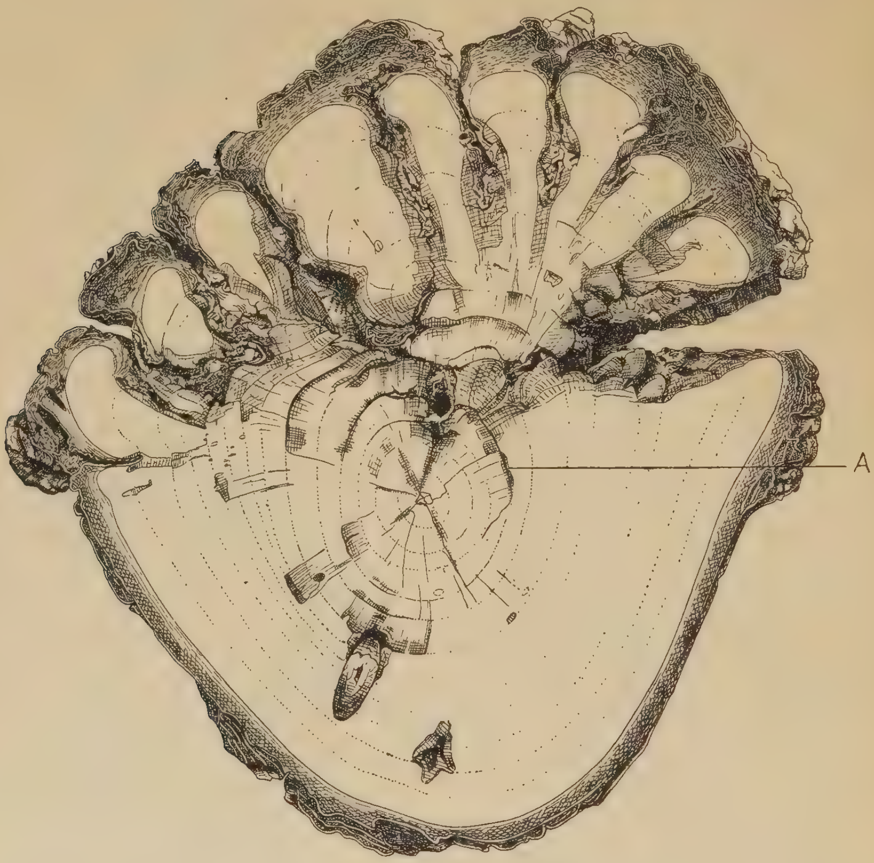


Fig. 4. Cross section of the stem of *Populus brabantica* Houtz. One of the infections took place as the stem had a diameter corresponding to the annual ring A. Overgrowing of the wound, though each year initiated, dit not succeed. The stem is excentric, the diseased side being thicker.
(Drawn by Dr. J. Y. v. Vliet).

in spring, after which a rent and later on a small canker is formed (fig. 3a).

The figure 4 shows the slight tangential spreading of the infection. When the stem had a thickness corresponding with

the annual ring A, the first canker wound was formed. Every year the tree tried to overgrow this wound, which failed however and a narrow fissure remained, still corresponding with the outer world.

In the apple canker, in a cross section, at a level with the wounds, we find a spreading in tangential direction corresponding with the annual rings, so more broadly extended. At a great distance from the canker a discolouration is no longer visible.

6. The bacterial cankers of the poplar show a thickening of the stem or branch on the side of the attack. The disc in fig. 4 is excentric.

   In *Nectria* canker the thickening usually takes place in the healthy part, just below the canker wound or sometimes as a „counterknob" in the part of the branch just opposite the canker.

7. In bacterial canker the strongest development of the parasite takes place chiefly in spring or in early summer. Artificial infection is difficult or impossible in the months of December until the end of February.

   The development of *Nectria* may also take place in winter. Artificial infection with *Nectria* on poplars is only possible in autumn and winter (see chapter III).

The stages of bacterial canker of the poplar described under 1. and 2. may be seen from April just before the developing of the leaves until the end of June, perhaps even a little longer. The white clots of slime, protruding from the lenticels, are found exclusively at the beginning of this period; later on, rents occur from which large masses of slime are flowing. This exudation is often brown and sticky.

Which factors determine the phenomenon described under 2a (swollen bark with rents, eventually without any exudation) or the one, described under 2b (clots of slime coming from the lenticels without any previous swelling of the bark) is still unknown. The behaviour of the different poplars in this respect is not always the same. If slime-clots occur, we find them for the greater part on one-year old lignified branches. On older branches we may find them next to the canker. The rents and fissures are much

deeper in thick branches on account of the greater thickness of the bark, the previous swelling being thus also less distinct. Great masses of exudation protrude from the rents and also from the cankers themselves; this slime too, is brown and sticky. In summer callous excrescences are found near the rents, which may be rather regular.

The reason why sometimes case 4a and sometimes case 4b occurs in the spreading is still unknown too.

Under 5 the spreading along the ,,red line" is described. Microscopically the ,,red line" shows a structure as shown in the figures

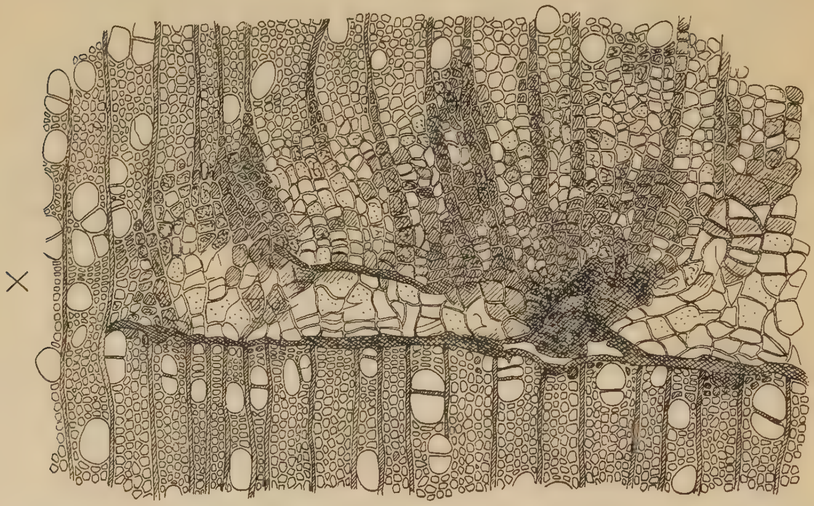


Fig. 5. ,,Red line" in the wood. An abnormal parenchymatous tissue has been formed instead of the normal wood. Cross section. × Limit of the annual ring.

5, 6 and 7. The oldest infected layer of wood has become a gummy mass after which instead of the normal wood a parenchymatous tissue has been formed. In the bark the sclerenchyma bundles have been infected and they are surrounded by a pathologic meristema (fig. 7). Further investigations must prove which factors are responsible for the occurrence of the abnormal parenchyma.

It was possible to point out bacteria in the sections with sufficient certainty. The sections were made in June 1938, from an externally healthy part of the branch. This part was situated

between an artificial infection of November 1937 and a secondary swelling of the branch which was formed in May 1938. The distance between the artificial infection and the secondary swelling was 17 c.M. The two lesions were connected by a „red line". The bacteria can be found in the cells of the medullary ray or the wood parenchyma, but also in the vessels, tracheids and fibres of the very young xylem. In the vessels of the xylem of the preceeding autumn the gummy masses are a great hindrance for determining the presence of bacteria. The cytologic work is still being continued however.

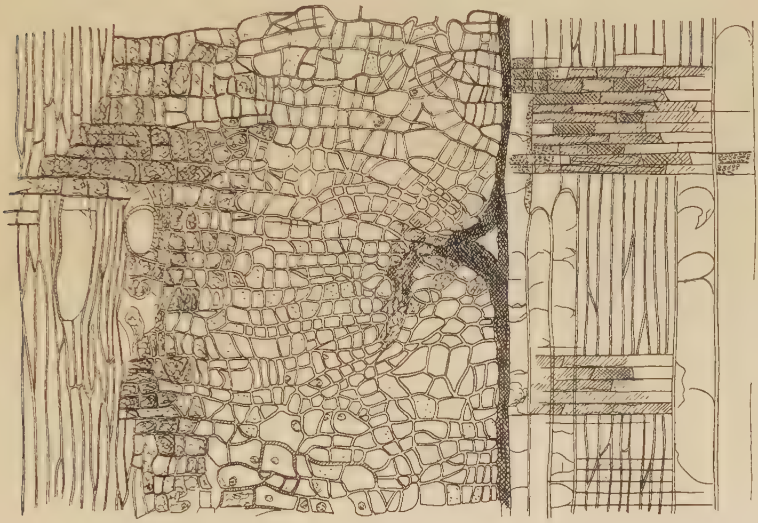
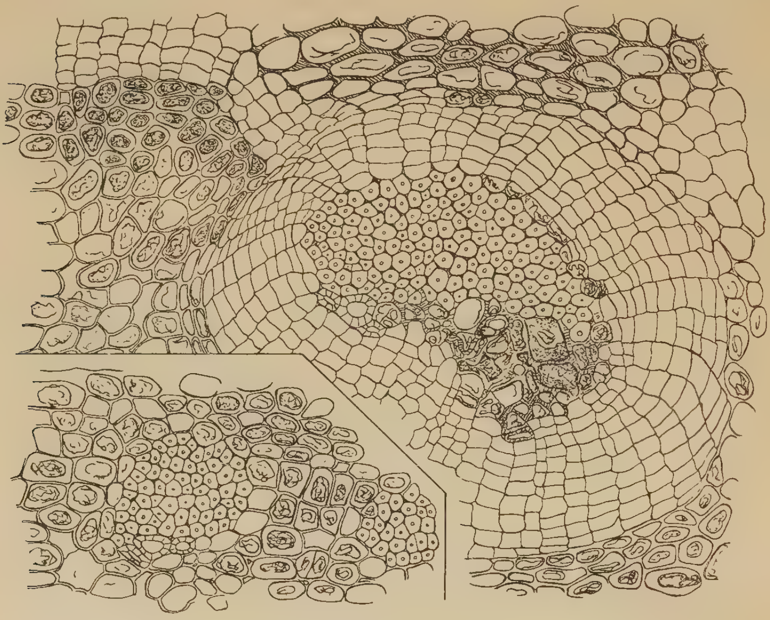


Fig. 6. „Red line" in the wood. Radial section.

Another fact to be examined is, for what reason in spring the „red line" suddenly comes into contact with the bark, causing it to burst. Perhaps the sugars, which in spring are mobilised in the tree, give rise to a highly increased development of the parasite, so that suddenly a mass of slime is formed. A fermentation of the sugars under gasformation is *not* caused by the parasite.

In conclusion we may add the following facts about the difference between *Nectria* canker and bacterial canker. Whilst *Nectria* calls

forth necrosis, it looks as though the bacterium has a direct hypertrophic influence on the tissues, which may be compared with the tumour-forming activity of *Pseudomonas tumefaciens* Smith et



a *b*

Fig. 6. „Red line" in the bark. *a.* Normal sclerenchyma bundle. *b.* Infected sclerenchyma bundle surrounded by a pathological meristema.
(Fig. 5—7 Drawn by Dr. J. Y. v. Vliet).

Towns.) Stevens, even though the formation of tumours is then very slight. The fact, that, in summer, the tree is able to fully resist the *Nectria*-attack, whilst in the case of bacterial attack tumour-like irregular ridges occur, is in agreement with this supposition.

§ 2. *The cause of the bacterial canker.*

The exudations, which in spring are flowing from the lenticels in the form of small, greyish white clots of slime, have been discovered for the first time in the Netherlands in the beginning of May 1936.

The slime was immediately applied with a needle to the branches of some poplars. Or after a dilution with water, the slime was injected with a syringe.

These crude inoculations resulted in small cankers of a very irregular structure after 8 to 10 weeks (Pl. I fig. 2). The next year (1937), near the cankers in some kinds of poplars, new slime-clots were found, which had come out through the lenticels. In other kinds a swelling of the bark around the canker occurred. Later on, this bark burst open and callus was formed along the rents (fig. 3c); hardly any exudation had come out. The branches on which the secondary slime-masses were found, died off in the end; young foliage having twice appeared on them, withered almost directly.

From the slime-masses lots of bacteria and other organisms can be isolated. At the very first appearance, when the slime is still pure white, we find bacteria exclusively. At this stage we have also the greatest chance of finding the pathogen among them. At later stages the slime is often brown and sticky. It then contains many yeasts and fungi (a.o. *Pullularia pullulans)*.

In order to make isolations the slime of newly appeared clots was diluted in sterile water. Herewith streaks were made on plates of carrot extract-agar or pepton-agar. The colonies were streaked on plates a few times more until a pure culture was obtained. Many strains thus came into culture, most of them yielding no results after inoculation however, as they apparently were saprophytes. At last two strains were found, which proved to be pathogeneous. Both isolations had been made by the author in France. The slime clots were taken from balsam poplars of the experimental nursery of the Station de Pathologie végétale à Versailles. After inoculation at Baarn on June 2nd. 1937 in 12 day's time these two strains brought out very strong symptoms on branches of different varieties of poplars. The branches died off and also the young shoots on thicker branches near the places of inoculation, withered. Some of the branches recovered and in the course of summer cankers were formed on the thicker ones. These had not the very irregular structure which was to be expected. Most of them were even very regular, varying however with the species. So they were not quite identical with the cankers obtained by crude

inoculation, but the further development must stil be awaited.

In May 1938 in the Netherlands some other strains were isolated, which proved to be pathogeneous also. [1])

The bacterial suspensions, used for the experiments, were taken from cultures on beerwort-agar or carrot-extract agar on which the bacteria had been growing for 3—5 days at ± 24°. The suspension was made with sterile tapwater and a common syringe was used; sometimes no suspension was made, but the bacteria were taken from the medium with a needle. The infected needle was stuck into the branch as deep as 1—2 m.m. In both methods some 10 pricks were made on one spot of the branch. No extra precautions were taken to sterilise the branch, before the injecting or the pricking took place. In the first experiments the bacterial suspension proved to be highly infectious. When the control-pricks were applied on the same branch as the inoculations, the former always became infected, apparently from the dripping of the bacterial suspension or by rain. Drops of suspension happened to fall on other branches which had not been wounded; they sometimes caused infections. In the later experiments better precautions were taken, so that the uncontrollable infections could no longer occur. The controlpricks applied on separate branches always healed normally.

The bacterium could be re-isolated from the branches above and below the place of inoculation and from withering young lateral shoots close to the place of inoculation.

The re-isolated bacteria-strain, when inoculated, caused a formation of canker again.

The bacterium is a short rodlet with polar flagella and without spore-formation. So, according to Migula's system it belongs to the genus *Pseudomonas*. The rents and fissures caused by it, induced us to call the species „*rimaefaciens*".

§ 3. *Bacteriological description of Pseudomonas rimaefaciens n.sp.*

For the determination the „Manual of methods for pure culture

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[1]) Probably the best results may be obtained when the twigs with the slime clots are collected during wet weather in early spring. The twigs must be kept for some days in a cool place (closed glass box in refrigerator).

study of Bacteria" bij the Society of American Bacteriologists was chiefly used. Two strains were investigated, one isolation and one re-isolation.

Pseudomonas rimaefaciens n.sp. is a motile rodlet with 1—3 polar flagella of 1.5 μ (0.6—2.4) length, occurring singly or in pairs. Gram negativ; not acid-fast. No spores are formed.

Wit fuchsin in water and carbol-gentian violet a deep staining is obtained; with analin-water saffranin a light staining.

Growth on various agar media:

beerwort	abundant, viscid.
peptone-glucose-saccharose . .	,, ,, .
peptone (Chassaing) . . .	moderate, dry, iridescent.
potato-extract	,, , slightly, viscid.
carrot-extract	,, , slightly, viscid.
white bean-extract	moderate to scanty; yellowish, green pigment.

Growth on gelatin media:

beerwort	scanty to moderate	sunken, liquefying.
pure	,, ,, ,,	

On other media which have been tried, the growth was scanty, without special peculiarities. On cellulose agar no growth occurred.

The stroke is cream coloured, filiform with lobate edge, sometimes beaded. On beerwort agar the development starts somewhat later than on other media where good growth occurs, but it increases very quickly and finally it is most abundant of all and very viscid.

Growth on synthetic agar media: with KNO_3 and 1% of glucose: abundant, viscid. When adding chalk or using only 0.4% glucose a yellowish green pigment is formed. On synthetic media with $(NH_4)_2PO_4$ and 1% of glucose when chalk is added the growth is: dry, iridescent.

Growth on potato: moderate, glistening, cream coloured, without special characteristics.

Stab-cultures in various *agar* media show only surface growth.

Stabcultures in gelatin: fairly rapid liquefaction, in three days infundibuliform, sometimes stratiform. In a forthnight ½—¾ of the gelatin in the tube is liquefied.

In liquid media: surface growth occurs while a sediment ap-

pears in a few days. Sometimes the whole liquid becomes turbid (especially broth), but always more so at the top layer (½ c.M.).

The liquefied gelatin shows the same phenomenon.

The shape of the colonies is round, convex, smooth; the microscopic structure is somewhat granular with a hyalin edge. As the bacteria are strict *aerobic,* colonies *in* the agar do not develop. Other tests (absence of growth under a coverglass on the plate and in stoppered bottles) also showed that the bacteria are *strict aerobic.*

Hardly any *yellowish green fluorescent pigment* is formed on the ordinary culture media. It may be seen on white bean extract agar (prepared from seeds of Phaseolus vulgaris L.) on diluted broth agar or on the synthetic medium mentioned above. It is always and very abundantly formed in a thin layer of a solution containing:

0.1% KNO_3
0.04% K_2HPO_4
0.02% $MgSO_4$

} with one of the following organic substances:

Sodium succinate		0.4%!
Calcium malate		,, !
Sodium salicylate		,, !
Calcium lactate		,,
(Sodium citrate		,,)
glycerol		,, !
mannitol		,, !
saccharose	1%,	0.4%
glucose		0.4%
laevulose		0.4%

From the basic solution KNO_3 can be left out to obtain the same results with urea, asparagin(!) and slightly with $(NH_4)_2PO_4$. In the latter two cases succinate must be present.

When chalk is added the results with glucose and laevulose are better.

The Ph always must be more than 7.5, during the experiment it may increase till > 9.

The optimum temperature for growth is 25° C. At 30° C the

growth is scanty; at 37° no more growth occurs. At 14° the development is very slow but is good in the end.

Letal temperature. The bacteria have been kept in a suspension in sterile tapwater. The suspension was made from a young culture on agar slant.

Thus the letal temperature was determined as 42°—48° C (See table I).

TABLE I

Temp.	time	growth on carrot extract agar, and on peptone agar	growth on beerwort agar
48° C	5 min.	none	none
46° ,,	15 ,,	(+)	—
46° ,,	5 ,,	++	(+)
44° ,,	50 ,,	+	—
44° ,,	15 ,,	+	—
42° ,,	50 ,,	++	—
40° ,,	50 ,,	++	+

Reduction of Nitrates could not be obtained, neither with peptone-KNO_3 under aerobic nor under anaerobic condition (no growth occurs in the latter case); neither in the ordinary synthetic media with KNO_3. Ammonia is not formed in the latter case, while it is always formed in precense of peptone, broth, gelatin or in natural media. Nitrite, when added, does not disappear.

So in the ordinary tests, according to the Manual of the S.A.B. no nitrate reduction takes place. However in the following solutions the nitrite reaction can be obtained very distinctly with the reagent of Griess Romyn (le Cosquino de Bussy 1936, pg. 35):

0.1% KNO_3, 0.04% K_2HPO_4, 0.02% $MgSO_4$ with one of the carbon sources: sodium succinate 0.4% or calcium malate 0.4%.

With sodium citrate, glycerol, mannitol, laevulose and saccharose the nitrate reaction does not occur regularly; with calcium lactate and sodium salicylate it is very uncertain; whereas it is absolutely absent with glucose or galactose.

No *Hydrogen sulfide* production.

A small amount of *indol* is formed.

Peptonisation of milk with alkali production (NH_3) is visible already in one day in the top layer. In 5 days the whole tube is peptonised. *Litmus* when added is *reduced* in 12 days.

The production of rennet or lab ferments could not be recognised, as coagulation was not obtained before peptonisation took place; nor by adding some peptonized milk to unsterilized milk. Because the tests in question have little evidential value, it must not be concluded from this that these ferments are absent.

Hydrolysis of starch was not obtained.

Several *carbon sources* have been investigated with the auxanographic method, KNO_3 serving as the nitrogen source. In table II the results are to be seen.

Gas production is never found. Experiments in fermenting tubes were made with glucose in peptone-water and in synthetic media.

Acid is formed when sugars are present.

Suitable *nitrogen sources* are: peptone, asparagine, urea, gelatin, glycin, nitrates and ammonium salts. Whereas caseine, albumin, leucine etc. do not occasion any growth in the auxanograms.

For the comparison of *Pseudomonas rimaefaciens* n.sp. and *Pseudomonas tumefaciens* (Smith et Towns.) Stevens, both bacteria were inoculated in young stems of *Pelargonium* and *Impatiens*. In 2—4 weeks *Pseudomonas tumefaciens* produced tumours on both plants. *Pseudomonas rimaefaciens* caused sunken areas on the stems of *Pelargonium*, whereas *Impatiens* showed a black discolouration of the vascular bundles more than 15 c.M. lower to the base. Large parts of the inoculated stems of *Impatiens* died. Later on all the plants of *Pelargonium* and the remaining parts of *Impatiens* totally recovered. The experiments were carried out in October and November.

In addition *Ps. tumefaciens* was inoculated into the twigs of various poplars. The results (Pl. II) agreed with those obtained by Smith (1911, pg 51, Pl. V and XXIII).

The pathogenicity of *Pseudomonas rimaefaciens* was not compared with that of other plant pathogens. From a bacteriological point of view it has many characteristics in common with part of them. The most striking peculiarities of *Ps. rimaefaciens* are its aerobism, the rapid liquefaction of gelatin, the rapid peptonisation of milk, the lacking of the capacity to split up higher carbohydrates,

(we remind here of some characteristics of *Pseudomonas tumefaciens* (Smith et Towns.) Stevens, which does not liquefy gelatin

TABLE II

Abundant	Moderate	Scanty	None
glucose	mannitol	laevulose	starch
saccharose		galactose	dextrin
glycerol		salicylate	inulin
succinates		lactates	maltose
malates		(arabinose ?)	lactose
citrates			rhamnose
oxalates			(xylose ?)
			salicin
			tartrates
			acetates
			formiate

and of *Bacterium (Pseudomonas) Savastanoi* var. *fraxini* Brown which hydrolyses starch and splits up several higher carbohydrates but does not liquefy gelatin.

CHAPTER III.

NECTRIA CANKER OF POPLAR

In the preceeding chapter apple-canker was taken for comparison, it being the best known form. In the poplar too, however, a *Nectria* canker may occur, which is similar to the apple-canker. Figure 8 gives instances of that. We notice the regular callous wall around the wound. Also the first signs of a spreading are visible in the circles of sporodochia of *Cylindrocarpon* outside the callous wall.

Nectria coccinea (Pers.) Fr. as well as *Nectria galligena* Bres. are found in these cankers. In far the greater part of the cases however it is *Nectria coccinea*.

Inoculations were made with both fungi; as no difference of infection was found with the two species, the further experiments were made with one and the same strain of *N. coccinea* only. The fungus was grown on malt-salep agar in Petri-dishes; in 3—4 weeks an abundant mycelium with conidia is formed there. This overgrown agar is cut into little blocks, which are put on a branch, the part with the mycelium placed downwards. In the branch 7 holes had been pricked beforehand with a stamping instrument, specially constructed for this purpose. The whole was loosely wrapped in a very thin layer of cellophane, tied up on the upper and lower side by adhesive tape.

In the control-inoculations a little block of sterile agar without any food-substances was placed on the 7 holes and a cellophane wrapping was applied to it. The use of agar without food-substances has the advantage of preventing saprophytes or *Nectria* spores, which happen to be present, to have an extra chance of growing.

The little control-wounds, when healed, always became 7 small callus knobs with a slight thickening of the branch around them.

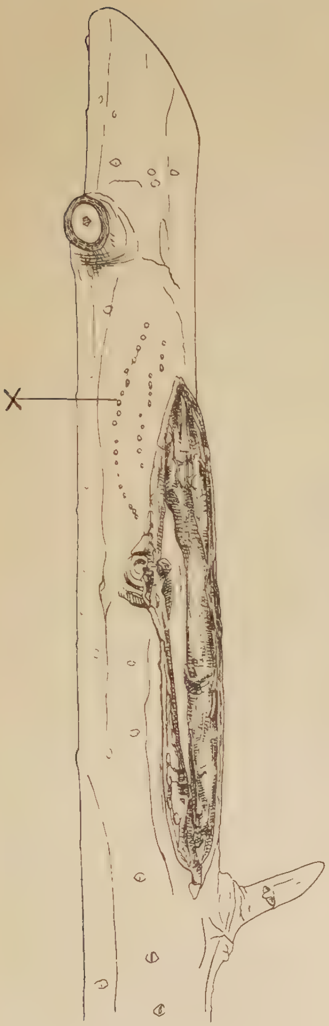

Fig. 8. *Populus brabantica* Houtz. Canker caused by *Nectria coccinea* (Pers.) Fr. Sporòdochia outside the callous wall (X).
(Drawn by Dr. J. Y. v. Vliet).

That is the reason why in the end the controls were left out. No special precautions as regards sterilising of the branch were taken.

Various other inoculation-methods were tried. The one used here, where the mycelium could grow inwardly through the little holes, attacking from there the bark and the wood, proved to be the most satisfactory.

Inoculations made from May till August during the period of strong growth of the tree, show no results at all; the wounds soon heal, though some difference with the controls may always be observed. The strongest infection is found with inoculations, made in September until the end of November, whilst January-March also yield good results.

All thin branches will die after the infection, without any callus formation occurring around the wound; the fungus attacks the whole branch. Sporodochia may be observed on the bark, especially in spring and autumn. In very susceptible varieties even rather big branches may die this way.

In the thick branches of non-susceptible trees the wounds will heal very soon in the first period of growth, after the fungus has been applied to them. The spreading of the fungus, before the callus formation started, depends more or less on the fact whether the inoculation took place in autumn or in spring, but it is in no way extensive. We then find sunken areas in the bark with dark discolourations.

In the thick branches of susceptible varieties a considerable extension of the wound is found; the bark shows sunken areas over a long distance of the branch and it sometimes even disappears altogether, so that the wood lies open to the view.

Around it, in the period of growth of the tree, provided the latter be in a good condition, a callous wall is formed. Sometimes this wound gradually closes itself during the successive periods of growth; often however the wound remains open with a callous edge. A canker is thus formed of a very regular structure, with smooth edges and resembling an apple-canker (fig. 8).

An annual concentric extension however, as found in the case of the spontaneous cankers, never occurred in the experimental cankers.

In a cross section a discolouration of the wood is still found at the outside of the callous wall. It generally has the form of a wedge. *Nectria* can be isolated again from this discoloured wood; so the fungus is actually present in a live form, but does not manifest itself externally. The development of the branch or stem is inhibited, but they are not killed by it. If, however, the tree gets into a bad condition, e.g. by suppression by the surrounding taller specimen, the fungus will be able to develop. The development is very rapid, the branch or stem dies and sporodochia will come forward everywhere. This also may be seen, when the tree is transplanted, so, *in all cases, where the entire growth is retarded.*

Up till now no perithecia of *Nectria* were found as yet on the inoculated parts; they may be found on very old, spontaneous cankers.

The above mentioned facts showed themselves, when making the investigations as regards the susceptibility to *Nectria* of 42 poplar species. These species in the years 1936—1938 were all inoculated at regular intervals during the whole year. Publications about the results will be placed annually in the periodical of the „Nederlandsche Heidemaatschappij", Arnhem. (First report Dec. 1937).

There are still other kinds of fungi, that cause the regular type of canker. *Botryodiplodia Penzigii* Petrak and Sydow, just like *Nectria,* was frequently isolated from poplar-cankers in the Nether-

lands. When inoculated, it calls forth the same symptoms as *Nectria*. The reaction is even stronger in general. The wood-discolouration occurring in consequence of *Botryodiplodia* is black-purple; in the case of *Nectria* it is red-brown. Inoclulation experiments with *Botrydiplodia* are running.

CHAPTER IV

COMBINED FUNGAL AND BACTERIAL CANKERS OF THE POPLARS

It is difficult to find pure *Nectria* cankers on „Black Italian" and „balsam" poplars in the Netherlands; they appear to occur only sporadically. Neither do we find many pure bacterial cankers. Nearly always the bacterial infection is followed directly by one or more fungi. In thinner branches, which sooner or later will die in consequence of the attacking, often species of the genus *Fusarium* are found. In branches which have already died altogether, *Cytospora* may occur. In thicker and stronger branches which remain alive after the attacking and begin to form cankers, in the Netherlands we very often find *Nectria*. In spring near the rents of the bacterial canker we then find the sporodochia of *Cylindrocarpon*. We may also find these on and around the irregular callus excrescences of older bacterial cankers. Here we find perithecia of *Nectria* as well.

All this makes it clear why in our country *Nectria* for a very long time was considered to be the chief cause of poplar canker. The exudations and in particular the clots of slime, indicating the bacterial character of the canker, can only be observed during a short time of the year and are actually only distinguishable in rainy weather.

It is remarkable, that the number of more or less parasitic fungi found in bacterial cankers, is comparatively so small in France. Of special importance is the almost total absence of *Nectria* there. So the bacterial cankers occur in a pure form, which simplifies the researchwork very much. [1])

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1) It is in this place, that the author wants to express her warmest thanks

Another striking difference between bacterial canker in France and in the Netherlands, is the very rare occurrence of the slime clots on the local variety of Black Italian poplars. (Régénérés de l'Ourcq; see page 14 under 2b.).

Whilst finding these slime-clots regularly in the Netherlands on cankered specimen of *P. brabantica* and *P. candidans* as the initial stage of new cankers, we find in France the swollen bark, as described on page 14 under 2a. The slime clots could be found however in France on the poplars of the balsam group, in the experimental nurseries of the „Station de pathologie végétale de Versailles", situated at Cuts near Noyon in the centre of the canker regions.

We need not wonder that this phenomenon which shows the nature of the canker so very clearly, was not known in France. The few balsam poplars, found here and there in the parks, are not exposed to the infection and consequently have no canker. The balsam poplars in the experimental nurseries at Cuts have only been growing there for some years and were not attacked by the disease until quite recently. Probably they did not show the clots of slime until 1937. Only by studying the canker both in the Netherlands and in France, the author became convinced that we have to deal here with a bacterial disease.

The exudations from the older cankers and from the rents near to them, had long been known in France.

As has been said already on the pages 20 and 23, these exudations however contain many yeasts and spores of fungi by the side of numerous saprophytic bacteria. On account of this it is not easy, not to say impossible, to isolate the pathogenic bacterium from them.

In England the clots of slime were known for a long time already, as the author heard from Mr. T. R. Peace of the Royal Forestry Institute at Oxford, even for some time be-

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to the Directors of the Dr. Chr. Buisman-fund, who made it possible for her to study the disease in France.

She also wants to express her gratitude to all those in France, who helped her in her work and who enabled her to visit the infected regions. Special thanks must be rendered for this to Prof. Dr. E. Foëx, directeur de la Station de Pathologie végétale au centre de Recherches Agronomiques à Versailles.

fore she found the clots of slime in the Netherlands. The material of *P. Eugenei*, sent by Mr. Peace, confirmed the supposition that the phenomena of the slime-clots in the Netherlands and in England, were identical.

The wood-boring insects cause a great deal of confusion in the study of bacterial canker. *Saperda carcharias* L. preferably lays its eggs in canker wounds. The young larvae at first, in order to feed themselves, bore tunnels closely under the bark, lying tangentially around the branch. When these wounds heal, all kinds of swellings are found, which are in no way connected with canker. Around the later tunnels which are bored deeper into the wood by the larvae, strong discolourations occur, caused by the penetration of all sorts of woodrotting fungi. An eventual „red line" is then no longer to be seen.

The larva of *Saperda populnea* L. begins by making closed round, gall-like swellings on the branches. If these begin to enlarge, rents are formed and a wound may then occur around the hole from which the young capricorn beetle has flown. The callous formations found then along the edges, make these infections look very much like cankers..

Lots of other closed nodes and healed wounds, all resembling bacterial canker more or less, may occur, especially in *P. nigra* L. and in *P. nigra italica* du Roi. The cause is still unknown.

SUMMARY

It has been proved by a detailed study of the disease called ,,poplar canker'', that there exist different forms of canker, caused by different parasitic organisms. One of these forms however is prevalent throughout the Netherlands, Belgium and the Northern part of France. It has been described in this publication as the ,,bacterial poplar canker''. By comparing this prevalent canker with the wellknown type of European apple-canker (the *Nectria* canker) the specific characteristics of this bacterial canker could be clearly brought to the fore.

These special symptoms are:

1. the exudations, which sometimes show themselves as clots of bacterial slime. They come out of the lenticels in the very first stage of the canker.
2. the irregular, crumbly structure of the callus excrescences.
3. the narrow fissures which are formed in the wood and in the bark.

The bacterium, isolated from the exudations, in the first stage of the canker, has been inoculated in the twigs and stems of different kinds of poplars. It reproduced the canker described above though the structure was less irrigular, as in the cankers, which were obtained by the inoculation of the crude exudation. The same bacterium has been reisolated from these artificial cankers; it had the same pathogenicity as the originally inoculated one.

The bacterium could not be identified with any other species already known. It thus was described as *Pseudomonas rimaefaciens* n.sp. Its bacteriological characteristics have been investigated. No other hosts as yet are known.

The variety *Populus brabantica* Houtzagers (,,Heeswijksche Witte''), *Populus trichocarpa* Torrey et Gray and *Populus candicans* Ait. proved to be very susceptible.

The sensibility of different species and varieties of *Populus* are being tried out.

Following these investigations the symptoms of different fungus cankers have been described and discussed. They form a more or less homogeneous type, which may be easily distinguished from the bacterial cankers. This type corresponds with the classical apple-canker caused by *Nectria galligena* Bres. The fungus canker of the poplar may be caused by *Nectria galligena* Bres. or *Nectria coccinea* (Pers.) Fr. or by *Botryodiplodia Penzigii* Petrak et Sydow, possibliy also by other organisms. Whereas *Nectria* may cause die-back, without the formation of previous cankers also.

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Fig. a.

Populus brabantica Houtz. (Heeswijk-sche Witte). Canker caused by *Pseudomonas rimaefaciens* n.sp.

Fig. b.

Experimental cankers on twig of *Populus brabantica* Houtz. after inoculation with bacterial exudation, directly taken from an other tree.

Populus alba Bolleana Lauche. Tumour formed after inoculation with a pure culture of *Pseudomonas tumefaciens* (Smith et Towns.) Stevens.

ANTAGONISM BETWEEN VARIOUS MICRO-ORGANISMS AND DIFFERENT SPECIES OF THE GENUS PYTHIUM, PARASITIZING UPON GRASSES AND LUCERNE

BY

A. VAN LUIJK

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INTRODUCTION

When investigations were being made about parasitism of various *Pythium* species on grasses, one of the experiments yielded very unexpected results. *Agrostis stolonifera* was inoculated with 4 different species of *Pythium*, partly in sterilized and partly in non-sterilized soil in glazed pots. *Pythium volutum* Vanterpool et Truscott proved to be highly parasitic in sterilized soil, *Pythium de Baryanum* Hesse however caused less damage.

In non-sterilized soil the parasitic influence of *Pythium volutum* was much weaker; the other 3 species of *Pythium* did not bring about any disease symptoms at all. The seedlings in the control-pots with non-sterilized soil remained healthy in the beginning; later on however damping-off appeared, caused by parasites occurring in the soil. The same thing happened in the pots with *Pythium mamillatum*. The seedlings in the pots with *Pythium de Baryanum* and *Pythium irregulare* however remained in a healthy condition.

On the one side *Pythium de Baryanum* and *Pythium irregulare* were not parasitic in ordinary soil, on the other hand the parasites occurring in the soil were not active when either *Pythium de Baryanum* or *Pythium irregulare* were added to the soil.

It is a well known fact that inoculation experiments in non-sterilized soil may give no results or less strong results than in sterilized soil; it is however a less generally known fact, that the addition of other parasites will counteract the original parasitic influence.

These results induced me to take up the question of antagonistic and synergetic influence between soil parasites and saprophytes.

The inhibiting influence of soil fungi and bacteria is frequently ascribed to competition to the food-substances available. When I began these experiments, some publications had already appeared

in which the idea of parasitic or toxic influence was put forward. For the literature concerning this subject I want to refer to the splendid survey by Waksman (1937).

There are certain difficulties as to the value of experiments with soil borne parasites in sterile and non-sterilized soil.

Inoculation experiments in non-sterilized soil will only then be convincing, when strong parasites are used. It may however be that the particular parasite or others are already present in the soil in question.

Experiments in sterilized soil on the other hand have the following difficulties: the high temperature alters different qualities of the soil; after some time organisms will invade the soil and may influence the parasite to be tested and the seedlings; thus in different sets of experiments different results may be obtained. Strong parasitism in sterile soil does not include the same rate of parasitism in untreated soil.

Some investigators (Hartley 1921, Pfältzer 1927) have added saprophytic fungi to the sterilized soil for their inoculation-experiments. These organisms counterbalanced the action of the parasite and kept its influence in bounds.

CHAPTER I.

METHODS AND MATERIAL

As a rule the first experiments were made in pure culture in vitro for both host and parasite. The same methods were followed in my former investigations about grass parasites. (1934)

In this class of experiments all casualties are being excluded. Of course it is not certain that parasitism in nature is of the same intensity. This however may be investigated later on sterilized and non-sterilized soil in pot-experiments or in gardenplots in different kinds of soil.

It is evident that antagonism first of all should be tried out in vitro.

The test-tubes used for these experiments were of the common size. They were partly filled with sieved garden soil. In order tc obtain a thorough mixture of both sand and humus particles, it was necessary to use rather dry, but not entirely dried soil. There should be equality of water capacity and of nutritional elements in the different tubes, as far as this is possible.

Sometimes water was added, sometimes a Knop solution or another mineral solution with addition of 0.5% saccharose.

In order to exclude sometimes the differences which must evidently exist in leaf-mold between the different tubes of one series, liquid medium and filter-paper were used of which the latter was folded in the form of an open cylinder. The seeds then were put on the top of this little bank of filter-paper.

As parasites *Pythium volutum* Truscott, *P. de Baryanum* Hesse, *Pythium intermedium* de Bary, *Pythium irregulare* Buisman and *Pythium mamillatum* Meurs were used in the case of grasses. (photo 1). *(Lolium annuum,* var. *Westerwoldicum, Festuca duriuscula, Agrostis stolonifera); Pythium de Baryanum* with lucerne.

For the latter the other species of *Pythium* were less pathogenic, *Pythium volutum* not at all.

As to the taxonomy of *Pythium de Baryanum*, I want to refer to my publication of 1934 b; Drechsler in 1934, 7 years after his preliminary publication has at last explained more in extenso what he means by *Pythium de Baryanum*. In my opinion this must be a type of *Pythium irregulare* Buisman with few protuberances on the oogonia. After Drechsler there is: ,,a close parallellism in every phase of development and morphology with the more abundantly and conspicuously digitate species *P. irregulare* and *mamillatum*." In consequence of Drechslers opinion the name *Pythium irregulare* should be changed into *Pythium de Baryanum*. Drechsler however points out more differences than agreements between his organism and that of Hesse.

As antagonists the following organisms [1]) were tested; *Phycomycetes* met with as parasites on grass roots and as saprophytes; *Pullularia pullulans*, found by chance; different fungi isolated from soil suspensions, from healthy patches of grass, occurring in plots with root-diseases.

Soil suspension was added to the seedlings and parasite; isolations were made and organisms selected and transferred with regard to their antagonistic power. *(Verticillium cinnabarinum, Mucor spec., Sporotrichum spec., Oospora spec., bacteria* and *amoeba)*.

Another principle was followed, namely that of finding thermostabile growth-inhibiting substances in liquid media (50 cc. Knop + 0,5 saccharose in Erlenmeyer flasks), after having exposed them to the open air or by inoculating them with gardensoil. This liquid was exposed 5 minutes to 102° C. and added to a culture of *Pythium de Baryanum*. In several of the flasks, the liquid contained growth-inhibiting substances. From the original flasks organisms were isolated. The fungus producing the largest quantity of the toxic substances was *Penicillium expansum* (Link) Thom.

The antagonistic organisms separately and in combination were

[1]) As many groups and genera of fungi have not been studied extensively, it was not possible to determine all the antagonists. A more detailed study of some of the related fungi would then have been necessary. It was not possible to save time for this kind of work.

tested with parasite and host together and with the parasites in culture. For the latter the culture liquid was either filtered through an E. K. Seitz filter, or heated up to 102° C. for 5 minutes.

Pythium spec, develops very abundantly in synthetic liquid media; for testing the growth of the antagonistic organisms against the parasites, the liquid was inoculated with an equal quantity of both organisms, transferred from cultures of the same age.

As it seems that agar sometimes absorbs growth-inhibiting substances, the original cultures have been made in Petridishes with a solution of 2% agar in water. This layer should be quite level. The *Pythia* grow very rapidly on this medium and at 25° C. the rim of the dish is reached in two days. At this moment the mycelium is in the best condition to be tested with antagonists.

The pieces of agar + mycelium were cut out by a small instrument, made by doubling up a piece of chromic nickel thread, ¾ m.m. thick and 10 c.m. long, the ends of which have been flattened. The larger part of the thread is interturned, the flat ends only remaining free. In this way a fork is formed which has a width of 4 to 5 m.m. between the two prongs and with this instrument exactly equal blocks of agar can be cut. For fungi like *Pythium* which cannot be obtained from spores, it is desirable to cut out a circular ribbon, some millimeters removed from the edge of the petri-dish, which ribbon is divided crosswise into equal parts. With other fungi, that can be obtained from spores, the whole plate of agar may be divided into equal parts by cutting it into two perpendicular directions. When taking the precautions described, it very seldom occurs that a difference in growth of more than 1 or 2 m.m. could be observed in parallel tubes of one series.

Besides, filtered or sterilized liquid media of the antagonists in pure cultures were added to hostplant and parasite. Finally the same experiments were made in soil in pots.

CHAPTER II.

EXPERIMENTS WITH GRASSES

§ 1. *Comparative infection tests with Pythium species on grass in sterile and non-sterile soil in pots.*

As has often been proved, the infection in sterile soil is much stronger than in non-sterile soil, through the influence of the antagonists. I am giving here a selection from a number of experiments:

Agrostis stolonifera and *Pythium volutum*.

Pots of 7 Liter contents, glazed, filled with sandy gardensoil and in the initial stage placed on a table outdoors, covered with a glass roof only.

At the end of the experiment the average dry weight per pot was determined.

sterile soil	17.31	gram	4 pots
,, ,, & *Pythium volutum*	3.96	,,	8 ,,
non-sterile soil	9.68	,,	4 ,,
,, ,, ,, & *Pythium volutum* . . .	6.57	,,	8 ,,

The difference between these series becomes clearer, when we take the relation to the controls in soil without organisms into consideration. In sterile soil with *Pythium* the dry weight is 22% of the control. In non-sterile soil the weight of the *Pythium volutum*-series comes up to 67,9%.

If the dry weight had been determined a month earlier, the difference would have been larger (photo 2). In the later stadia *Pythium* does no longer do any harm and the plants which were

not killed, develop in a normal way. Even the few plants of the *Pythium* series begin to „shoot", probably because they have plenty of nutrition at their disposal.

These differences must be brought back to other organisms living in the non-sterilized soil, or may be to the competition for nutritional substances.

§ 2. *Comparative infection tests with pure cultures of grass in testtubes (Festuca duriuscula) and combinations of Phycomycetes.*

The numerous observations about differences in results between inoculation experiments in sterile and non-sterile soil, made it necessary to prepare experiments on combinations of different organisms. In order to avoid influences of occasional organisms, the first experiments were taken in pure culture in tubes. Different *Phycomycetes* were selected as parasites and antagonists.

Three parasitic spec. of *Pythium (P. de Baryanum* Hesse, *P. irregulare* Buisman and *P. volutum* Vanterpool et Truscott) and the following saprophytic *Phycomycetes: Pythium spec., Pythium torulosum* Coker et Patterson and *Mortierella spec.*

Ten testtubes each with 10 seeds and 10 cc. gardensoil and 3 cc. sterile water, control after one month.

Germination of seeds in %

control	82
Pythium spec.	86
Mortierella spec.	85
Pythium torulosum	77
Pythium irregulare	76
Pythium de Baryanum	61
Pythium volutum	45

Pythium de Baryanum and *Pythium volutum* proved to be the strongest parasites and were chiefly used for further experiments on antagonism.

Pythium volutum with combinations of the other 5 Phycomycetes; germination after 1 month:

Pythium volutum		100 seeds			45% germination
,, ,,	+ 1 fungus	500 ,,	5 combinations		53% ,,
,, ,,	+ 2 fungi	1000 ,,	10	,,	67.3% ,,
,, ,,	+ 3 ,,	1000 ,,	10	,,	71.7% ,,
,, ,,	+ 4 ,,	500 ,,	5	,,	78% ,,
,, ,,	+ 5 ,,	100 ,,	1	,,	78% ,,

The germination-power increases by the addition of the numbers of fungi up to four, only 10.8% of the damage of *Pythium volutum* when alone then being done. The antagonistic influence against this fungus by the 4 or 5 others together is thus 89.2%.

By these experiments it has been proved that it is possible to inhibit the parasitism of *Pythium volutum* through addition of other fungi.

§ 3. *Comparison of antagonistic influence of parasites and saprophytes. (Festuca duriuscula).*

It seemed probable that saprophytes would have more antagonistic influence than parasites mutually. The following table shows the reverse.

TABLE I.

			(germination)
Pythium volutum only		100 seeds	45%
with 1 parasite,	3 combin.	300 ,,	57%
,, 2 ,, ,	2 ,,	200 ,,	66.3%
,, 3 ,, ,	1 ,,	100 ,,	72%
,, 1 saprophyte,	2 ,,	200 ,,	47%
,, 2 ,,	1 ,,	100 ,,	58%

It is clear now that parasites may even be stronger antagonists than saprophytes.

With all combinations of 2 fungi there were only 2 cases, in which the damage done was stronger than by each of the components separately (in both cases a parasite + a saprophyte).

Stimulation of germination may be observed in this series, but

the difference with the controls is not so large that we can speak of a significant one. Probably there is an acceleration rather than a total increase of germination-power. In order to decide this matter we had to state this increase at different dates. Eleven days after the sowing the controls showed 55.9% germination; when *Pythium* spec. and *Mortierella* spec. were added, there was an increase of germination of 10.8%. As 10.8% was four times as large as the standard error the difference was significant. Thus we must assume, that under the conditions present the two fungi have accelerated the germination of the *Festuca* seeds.

On page 1 I exposed a hypothesis that parasitic fungi might annihilate the noxious influence between them. The experiments then taken made it clear that the results could not be explained by competition in nutrition. Notwithstanding the presence of many organisms in that soil, parasites were active.

The addition of one parasite (either *Pythium de Baryanum* or *Pythium irregulare)* however kept the grass in a healthy condition. It seemed probable to think here of parasitism or growth-inhibiting influence.

The increase of antagonism combined with increase of number of organisms in the pure culture experiment however, could be explained by the competition factor as well as by the growth inhibiting factor. This problem was attacked in a different way, and along other lines.

§ 4. *Influence of filtered and sterilized media of Pullularia pullulans (de Bary et Löw) Berkh. on the growth of Pythium de Baryanum Hesse.*

The first experiments concerning this question were taken with *Pythium* species. These proved to contain growth-inhibiting substances.

On the other hand *Pullularia pullulans* in these experiments had a much stronger influence than *Pythium* spec. mutually. *Pullularia* was thus selected for the study of growth-inhibiting substances. It develops very rapidly in Knop solution + 0.5% saccharose forming merely yeast-like conidia, during the first period. The liquid is then cloudy. When temperature however is either too high or too

low, small colonies of mycelium are being formed, first yellow brown, up to black.

Filtrates were prepared:

1. from one month old cultures grown at 4—15° C.
2. same developed at 14—21° C.

The culture liquid was filtered, first through filter paper, then through Seitz E.K. filter. They were transferred with sterile pipettes to culture-tubes (15 cc). Several dilutions were prepared by adding quantities of the same culture-medium.

Pullularia was tested on its antagonistic influence upon *Pythium de Baryanum*. (photo 4).

TABLE 2.

Influence of filtrate of *Pullularia* on the growth of *Pythium de Baryanum*. Measuring of vertical growth 5 days after the inoculation of *Pythium*.

Dilution of filtrate	Pullularia culture from lower temperature		Pullularia culture from higher temperature	
	medium height of fungus in mm.			
	contr.	filtrate	contr.	filtrate
1/1	45	3	45	35
1/2	47	3	46	43
1/4	44	16	44	45
1/8	45	34	45	47
1/16	44	38	44	46
1/32	46	46	—	—

Every series 3 controls to every dilution. (Later on a smaller quantity proved sufficient). It would seem probable that the temperature at which the culture has developed, has influence upon the toxicity of the metabolic products. Other experiments however proved that the stage of development is the chief factor; the cultures grown at a lower temperature were but little developed, but exactly at the point of the highest production of toxic substances, whereas the ones grown at higher temperature where past the stage of optimal development.

Several series of *Pythium* in Knop solution were mixed with

boiled culture-liquid of *Pullularia;* I could prove here that the toxic substances were neutralized in their influence when exposed to 100° C. The height of the growth was practically the same as in the controls. The thermolability was further proved by testing several dilutions of boiled and non-boiled filtrate.

Growth Pythium: Controls 48—56 mm. height (6 days)

	Dilutions: 1/1	1/2	1/4	1/8
Filtrate boiled	48 mm.	48 mm.	51 mm.	50 mm.
„ unboiled . . .	0 „	15 „	41 „	50 „

The difference between the influence of *Pullularia* cells themselves and the metabolic products were tested as follows:

Pullularia cells were separated from their metabolic products by filtering. A suspension of these cells in Knop-saccharose was then heated in boiled water. In this decoction *Pythium de Baryanum* was inoculated. Growth of *Pythium de Baryanum* was measured in the different dilutions:

Control 42—46 mm.

1/1 43 mm.	1/2 62 mm.	1/4 61 mm.	1/8 62 mm.
1/16 58 mm.	1/32 56 mm.	1/64 52 mm.	1/128 41 mm.

The addition of *Pullularia* decoction showed stimulation in nearly all the dilutions. Similar results were obtained in other series.

From these experiments we may draw the following conclusions: *Pullularia pullulans* forms growth-inhibiting substances present in the filtrate; they are thermolabile; the substances which stimulate the growth are thermostabile and are probably chiefly present within the cells.

Some other observations could be made: The strain of *Pullularia,* which was tested, was rather inconstant as to its formation of growth-inhibiting substances. (This phenomenon is also mentioned by various other investigators as Fleming for his *Penicillium,* (1929) and Clutterbuck and others (1932). The same may be said of *Penicillium expansum* mentioned in the last chapter of this publication. The toxicity varied with the stage of development (at 15—20° the optimum was between 3 and 6 days). The highest toxicity was reached with a culture growing at 23° C. The growth

of *Pythium* in the *Pullularia* filtrate was inhibited at a dilution of 1/8 (culture of six days old).

Furthermore it was evident that the toxicity of the filtrate had no connection with the pH, the latter being within the range in which *Pythium* grows well.

The filtrate did not seem to change its characteristics very easily. When there was no growth of *Pythium* after one month, the re-inoculation in undiluted filtrate did not give any result either. In dilution 1/2 some development was observed however.

By other experiments a still smaller alteration was proved.

The mycelium of *Pythium* is probably killed by the toxic substances. The latter may be absorbed by kaolin.

§ 5. *Infection experiments with Pythium de Baryanum on germinating grass seeds in tubes with addition of Pullularia filtrate.*

Though in infection experiments with a combination of *Pythium* and *Pullularia* an attenuation of infection was determined, this did at first not occur when instead of the antagonistic fungus its filtrate was added. Probably the toxic substances were absorbed either by the soil particles or by the filterpaper. We therefore selected filtrates with the strongest inhibiting power on *Pythium* in pure culture only. From the different series which showed results, I can only mention the following: *Pullularia* filtrate from 3 days old culture, grown at 25° C.; inhibition of *Pythium*-growth up to 1/8 dilution. Cultures of *Lolium* in tubes, Knop-saccharose + filterpaper, filtrate undiluted. Infection with *Pythium* in 2% agar from Petri-dishes. Every tube inoculated with cuts of 5 mm. length and 2 mm. thickness. Inoculation four days before the seeds were brought into the tube. (photo 6).

The experiment was composed of 3 series of 12 tubes each:

with filtrate, inoculation with *Pythium de Baryanum*
,, Knop-sacch., ,, ,, ,, ,, ,,
,, ,, ,, , no inoculaton.

10 days later the percentage of germinated seeds was ascertained and the total length of the plants in each tube measured.

TABLE 3.

	Germination	Average length of seedlings pro tube
Inoculation in filtrate	68,3%	297,5 mm
„ „ Knop-sacch. . .	6,7%	14,8 „
No inoculation	70,0%	296,0 „

It has been proved that the rotting of grass seedlings may be inhibited by products formed by another fungus. Neither the seeds, nor the seedlings were damaged by these substances. The results remained the same at a later stage.

§ 6. *Experiments on antagonism in pots and large tubes (Simplicia tubes) under non-sterile conditions in the open air.*

It was to be expected that experiments in pots in non-sterile conditions would show differences; the external conditions will hardly ever be the same in the subsequent series. As *Pullularia* filtrate showed no antagonistic effect, the experiments were continued with *Pullularia* cultures. Positive results were obtained in the following case: Disinfected seeds of *Lolium annuum* var. *Westerwoldicum*, were inoculated in large tubes with *Pythium de Baryanum*. The soil was kept rather dry. Tubes were placed in the open air only covered with a glass roof.

Each series 3 tubes, each with 20 *Lolium* seeds. Inoculation with *Pythium* from a Petri-dish with 2% agar in water, culture 7 days old. 10 cc. of a culture of *Pullularia* in Knop-saccharose was added. After a month the grass was cut off and the dry weight of stems and leaves measured. (photo 5).

controls — average dry weight pro tube . . .	1650 mgr.
Inoculation Pythium „ „ „ „ . . .	760 mgr.
„ „ + Pullularia „ „ „ . . .	1637 mgr.

Antagonism *Pythium mamillatum* against *volutum* on *Agrostis stolonifera* treated in the same way gave similar results. (Every series 2 tubes). (photo 3).

controls	—	average dry weight .	445 mgr.
Inoculation Pythium volutum		,, ,, ,, .	12,5 mgr.
,, ,, ,,	+ Pythium mamillatum	average dry weight	260 mgr.
,, ,, ,,	mamillatum	. . . .	372 mgr.

A strong antagonistic influence of *Pythium torulosum* on *Pythium de Baryanum* was developed in a series of seedlings of *Lolium*, cultivated in wide tubes. The controls showed 85% germination after 10 days. Series with *Pythium* 12% only, *Pythium torulosum* only 76% and *torulosum* and *de Baryanum* combined 75%.

The results with *P. torulosum* were in general most divergent. Both antagonism and synergism were observed. In non-sterile conditions *Pythium* species can show a strong antagonism mutually, which however varies after conditions (from 0% till about 100%).

One example may be cited from series with *Agrostis stolonifera;* controls dry weight 440 mgr., with *P. volutum* 15 mgr., with *P. volutum* and *P. torulosum* 265 mgr.

From these experiments we may conclude that *Pythium* spec. may be antagonistic mutually in non-sterile conditions. The results are however strongly influenced by conditions. Practical application of these fungi cannot be expected.

CHAPTER III.

SELECTION OF ANTAGONISTIC ORGANISMS

§ 1. *General considerations.*

It seemed desirable to continue the investigations with virulent antagonists obtained by selection. *Pullularia pullulans* was found accidentally: other organisms would probably be detected in places where the parasite showed little influence on the host. It is evident that in cases of strong parasitism the antagonists did not develop sufficiently.

This work was started in 1935: an analysis of soil-organisms was made from a healthy grass-plot. The results were encouraging right from the beginning.

Agrostis stolonifera inoculated with *Pythium volutum* showed 92% infection, 8% seedlings remaining healthy. (photo 8). When however a drop of suspension of the soil to be tested was added, 52% seedlings remained healthy. It was proved that no parasites were present in the soil suspension; the whole influence was thus restricted to antagonistic organisms. In the different tubes to which soilsuspension was added the results were, for instance in one series of 20 tubes, that the 10 seeds in three of the tubes all germinated and remained healthy. In another tube of this series, 9 seeds germinated, but 8 of these showed infection by *Pythium volutum* and so on. Another series with *Lolium annuum* var. *Westerwoldicum* and *Pythium irregulare* as a parasite gave similar results. The soil suspension proved to contain only a few light parasites. The strong parasitic power of the *Pythium* developed only in a glass house, where the temperature often amounted to over 25° C.

§ 2. *Isolation of organisms from the soil suspensions.*

These were isolated from the tubes in which the strongest antagonism developed. Selection was then continued several times. In table 4 and 5 we find instances of the antagonistic effect of several species and of combinations. Only one organism showed no antagonistic effect. The strongest antagonist was a fungus without fructifications, but which proved to be of a parasitic nature towards the grassplant; so were several species of *Fusarium*. *Gliocladium* a severe antagonist in the case of grass, was parasitic in the case of lucerne. Parasitic organisms were later on eliminated.

TABLE 4.

Antagonistic influence of separate selected organisms against *Pythium volutum* on *Agrostis stolonifera*.

Series of 10 tubes each with 10 seeds.

number of healthy plants after	7 days	11 days	22 days
Control	25	45	88
Pythium volutum	9	8	19
P. vol. + Papularia spec. 1 . .	6	15	18
,, ,, + bacterium 13175 . . .	14	25	34
,, ,, + Papularia spec. 2 . .	21	26	28
,, ,, + Sporotrichum spec. . .	22	37	49
,, ,, + Gliocladium spec. . .	24	39	39
,, ,, + Bacterium 13177 . . .	25	30	36
,, ,, + Mycelium sterilium . .	27	41	59
,, ,, + Pullularia pullulans . .	27	36	49
,, ,, + Penicillium spec. . . .	28	37	35
,, ,, + Verticillium cinnabarinum	33	48	45
,, ,, + Amoeba + bacterium .	40	52	47

§ 3. *Combination of antagonists.*

Different strong antagonists were compared with combinations of other antagonists.

In the 3 control series with *Pythium volutum* only, there were only slight differences. There was no combination however, which

proved to be entirely satisfactory, so that no results in non-sterile conditions could be expected. (photo 8).

TABLE 5.

Agrostis. Series of 100 seeds.

number of healthy seedlings after	4 days	6 days	9 days	26 days
Control	8	42	73	86
P. volutum	1	6	20	21
,,	4	14	18	16
,,	0	2	16	17
P. vol. + Verticillium cinnabarinum	5	38	52	50
,, ,, + Amoeba + bacterium	8	42	51	57
,, ,, + Mycelium sterilium	5	41	47	41
,, ,, + Gliocladium spec.	16	62	76	63
,, ,, + Verticillium + Amoeba + Bacterium	7	47	61	49
,, ,, + Verticillium + Mycelium sterilium	13	54	55	41
,, ,, + Verticillium + Gliocladium	21	66	79	63
,, ,, + Verticillium + Amoeba + Bacterium + Myc. ster.	7	33	49	33
,, ,, + Verticillium + Amoeba + Bacterium + Gliocladium	18	66	79	72
,, ,, + Verticillium + Mycelium sterilium + Gliocladium	12	41	55	37

§ 4. *Stimulation of growth of the host-plant.*

Stimulation of growth of the host had been observed in several of the foregoing series of experiments; the same influence had been pointed out by us in 1934 (van Luijk 1934). The development of the seedlings in tubes with soil suspensions and Pythium was greater than in tubes without any fungi or bacteria (photo 8).

Organisms isolated from these suspensions produced the same stimulation.

The best stimulant was selected in the following way:

From one test tube, in which the stimulation was the most intense, one droplet of suspension was transferred to ten other tubes (+ ten controls) with seeds of *Festuca duriuscula*.

The figures illustrating the germinating power (after resp. 8, 13, 18 and 27 days) were 1, 32, 61 and 78 without and 8, 60, 81, 91 with addition of soil suspension.

With a *Mucor* spec., isolated from the twice selected suspension, an equal series was prepared. The figures are the following: germination without *Mucor* (after 2, 12, 15, 23 days) 3, 21, 43, 93, with *Mucor* 9, 48, 68, 94.

A combination of *Amoeba* (which cannot be cultivated purely) with a white and a yellow bacterium were tried upon *Agrostis stolonifera*.

Every series 12 tubes, every tube 10 seeds.

The dry weight of the seedlings was established after one month:

control	35 mgr.
amoebe + yellow bact. . .	60 mgr.
,, + white ,, . .	82 mgr.

P f e i f f e r (1934) points out a considerable influence to fungal invasion in the seed coat of *Symphoricarpus racemosus;* she is of opinion that the permeability for water and the germination both are stimulated. It may be that the same thing occurs in the grass seeds, which in this case had been harvested during a very dry period. In non-sterile conditions the germination was excellent. In a wet period however the permeability may be increased during the period of seedripening. This factor however cannot explain the accelerated growth of the seedlings.

§ 5. *Experiments with Medicago sativa.*

We were in need of another test-plant, because the germination power of *Agrostis stolonifera* decreases during the summer. We found lucerne very suitable, the more so, as *Pythium de Baryanum, P. irregulare* and *P. mamillatum* were all more or less pathogenetic to this plant. *P. volutum* did no harm. Most of the experiments were made with *P. de Baryanum*.

From U.S.A. a damping off has been described for Alfalfa by

different *Pythia* (Buchholtz 1935, Buchholtz and Meredith 1938). From Mauritius the same phenomenon was described in 1924. Klinkowski and Lehmann mention *P. de Baryanum* in 1937.

In testtubes *Pythium de Baryanum* attacks lucerneseedlings up to 100%, in sterile soil in pots 90—100%. The most important factors are: high temperature (25° C.) during part of the day, inoculation with two or 3 days-old cultures, and sowing 3 days after inoculation.

Combinations of already selected organisms were tried out; one example is mentioned here.

Lucerne cultivated in testtubes with Knop solution and filter paper, seeds previously germinated on 2% agar in water, 5 plants pro tube. Series of ten tubes.

Combination of *amoeba* + *bacteria, Bacteria* 5 and 7, *Verticillium cinnabarinum, Verticillium cinerescens,* and *Pullularia pullulans.* In other series one of the components was eliminated, *Pythium* and antagonists were put into the tubes one day after the seedlings. Temperatures minimum 10—16° C. and maximum 18,5—26,5° C. Results: Controls healthy; series with *Pythium* 48 on 50 seedlings diseased or dead. The number of the parasited plants was reduced to 27, when 6 antagonists were added. Addition or suppression of one organism, made little difference, except for the addition of *Mucor* spec. to the other 6 antagonists which cut down the number of parasitized plants to 9.

Mucor thus showed a strong inhibition on the parasitism of *Pythium.*

By suppressing *Bacterium* 7 from the combination however, the number of germinated seeds increased to 42.

§ 6. *Selection of antagonists as to strongest growth inhibiting substances.*

From the investigations with the selected microorganisms it appears, that several of them are capable of reducing the attack of seeds and plants by *Pythium* species and that sometimes they even fully inhibit these attacks. Hereby combinations of several organisms together may yield better results than each of the com-

ponents of the combination separately. When growing plant, parasites and antagonists together in pure cultures, no constant results are obtained however. One fungus or one bacterium or a combination of 2 or more organisms may be capable of inhibiting partially or wholly the attacking by *Pythium;* in other cases the same organism or combination can be fully or almost fully ineffective. Under nonsterile conditions we have greater chance of finding these contradictory results than in pure culture.

Organisms might be found however, so strongly antagonistic, that even under unfavourable conditions, they may yet be able to inhibit the attack by a parasite. The principal thing is to discover the strongest antagonists and this cannot be done by following the method, used for the two preceding experiments. When in one and the same experiment 2 organisms will both fully inhibit the attacking by the parasite, the one can yet be much more strongly antagonistic than the other. This cannot be proved however by this experiment.

In most cases the antagonistic action is certainly based on the formation of inhibiting substances, which having dissolved in the soil-moisture, have spread all over the soil.

When trying to discover the organism with the greatest antagonistic capacity, we must first of all find out which organism will form the inhibiting substances in the highest degree. This can be done by growing the organisms to be investigated in liquid media, which then may be sterilized or filtered. After this the parasite can be grown in various dilutions of these liquid media. The filtering of the liquid medium will prove to take up a great deal of time when we want to compare numerous organisms. It is much simpler to sterilize the medium, but the objection against this is, that the thermolabile substances are eliminated then. In the case of filtering on the other hand these substances are eliminated, which are absorbed by the filters. It was found out in the earlier experiments with *Pullularia pullulans,* that the inhibiting substances of this fungus were thermolabile. As appears from the literature about antagonism however, the thermostabile toxins are not rare either. So it was thought advisable first to experiment with thermostabile substances in the manner indicated.

First a series of all kinds of organisms, which I had in culture,

were compared. They were grown in Knop + 0.5% saccharose in Erlenmeyer flasks. After 21 days 10 cc liquid medium out of each flask was put into a tube and sterilized at 102° C. for 5 minutes. After that all the tubes besides 2 controltubes were inoculated with *Pythium de Baryanum*. The growth in height in the tubes was compared after 4 days.

TABLE 6.

Sterilized liquid medium of	Vertical growth of Pythium in mm.	Sterilized liq. med. of	Vertical growth of Pythium in mm.
Control	50	Bacterium from lucerneseed 4	40
,, ...	51	Pythium de Baryanum .	40
Verticillium cinerescens .	0	Gliocladium Vermoeseni .	41
Bacterium 4	0	Sporotrichum spec. . .	42
Cylindrocarpon sp. with bacterium	0	Gliocladium spec. . . .	43
Bacterium 7	7	Mortierella longipes . .	43
Mortierella pusilla vinacea	12	,, echinulata . .	46
Verticillium cinnabarinum .	17	Bacterium from lucerneseed 55	48
Gliocladium deliquescens	20	Pythium torulosum . .	48
,, nigrovirens	22	Botrytis from Aster . .	50
,, catenulatum	26	Sordaria from oak . . .	50
Mortierella pusilla atra .	30	Mucor spec.	50
Gliocladium roseum . .	34	Pythium spec. from grass	51
Bacterium 5	35	Pythium mamillatum . .	53
Pythium irregulare . .	36	Bact. from lucerneseed 51	55
Gliocladium luteolum . .	36	Mortierella isabellina . .	55
Pythium volutum . . .	37	,, pusilla . . .	55
Bacterium from lucerneseed 53	40		

Verticillium cinerescens Wollenweber and *Bacterium* 7 were among the best of the selected antagonists in the experiments with grass, lucerne and *Pythium* already. Now it is evident, that both will form the thermostabile inhibiting substances in a very high degree. Some further experiments were made yet

with those two organisms and an attempt was made to obtain a higher inhibiting effect by alterations in the composition of the liquid medium. By this it was effected that even in the case of diluting the liquid medium to 1/3, the growth of *Pythium* was yet fully inhibited.

In the meantime another new selection series was prepared. A number of Erlenmeyer flasks with Knop and 0,5% saccharose were placed in different spots in the Laboratory, in the hothouse and in the garden for an hour. In other flasks a bit of leaf-mold or soil was put from different parts of the garden. After some days, 10 cc of the liquid medium was taken from the flasks in which organisms had already clearly developed and it was poured into tubes and sterilized. In this experiment the piece of agar with *Pythium* was not immersed in the liquid, but it was put on the top of a piece of filterpaper (see pag 49) for the method).

In the former experiments with filtrates or sterilized liquid media it always was proved that the inhibiting effect was much slighter, when grass or lucerne were also present. An important factor here may be, that with *Pythium* only, the fungus was immersed with a column of liquid, 5 to 6 cm high. In the cultures with lucerne or grass the inoculation-material is found in the air on the top of filterpaper or soil. This may have a considerable influence on the resistance of the *Pythium* against the inhibiting substances; and yet another factor, causing a difference, may be the absorption by filterpaper or by soil. In the method followed here it could only be made certain whether a growth occurred or not; a reliable criterion for partial growth-inhibition was lacking. As the purpose was to obtain a full inhibition of the growth, even in the case of stronger dilution if possible, this was of no consequence however.

The liquid medium from many flasks showed a distinctly inhibiting effect already after a 3 or 4 days growth when the first control took place. Of 2 of these this effect was so strong already, that all growth of *Pythium* was inhibited.

Series of dilutions were made from the liquid out of these two flasks, when the cultures were 7 days old. In none of the concentrations 1/1—1/16 *Pythium de Baryanum* was able to grow. A new series was made of 12 days-old cultures and the diluting

was continued to 1/32. Here too in the strongest dilution the growth of *Pythium* was yet fully inhibited.

I now tried to find out which organisms with such an unexpectedly strong inhibiting capacity these two flasks contained. From one of them, No. 9, I isolated a *Penicillium*, a *bacterium* and 2 non-fructifying fungi. In a comparative experiment the *Penicillium* proved to be the originator of the inhibiting substances. These organisms had been found in one of the rooms of the laboratory.

The second flask, nr. 18, was inoculated with a grain of vegetable mould. In it were found a *Penicillium* and a *bacterium*. Here again it was the *Penicillium* which formed the inhibiting substances.

CHAPTER IV.

EXPERIMENTS WITH PENICILLIUM EXPANSUM (LINK) THOM AS ANTAGONIST

§ 1. *Inhibiting effect of the metabolic substances of Penicillium expansum versus Pythium.*

In the course of these investigations it was impossible to investigate extensively the systematic place of the different antagonistic organisms. The *Penicillium* however was thoroughly studied by Jhr. van Beyma thoe Kingma in the „Centraalbureau voor Schimmelcultures."

It proved to belong to the group *Penicillium expansum*. A type of this group is mentioned as antagonistic organism to *Sclerotinia fructicola* (Machacek 1928).

TABLE 7.

Sterilized liquid medium of *Penicillium* 9 and *Penicillium* 18. Different strains of separate colonies obtained by sowing. *Penicillium* cultures 3 weeks old. Growth of *Pythium* in tubes 6 days after transferring.

concentr.	9	9b	9c	9d	9e	18a	18b	18c	18d
1/2	0	0	0	50	0	0	0	0	0
1/4	0	0	0	50	0	0	0	0	0
1/8	0	0	0	50	0	0	0	0	0
1/16	0	0	22	50	0	0	0	0	0
1/32	0	35	37	50	26	0	0	0	0
1/64	0	50	50	50	50	0	6	0	0
1/128	13	50	50	50	50	0	27	21	30

4 controls, 50 mm. each.

Both strains were sown out; some separate colonies were grown and compared as to their inhibiting effect. In these and the following experiments, *Pythium* was inoculated once again in the usual way at the bottom of the tubes. (table 7).

The separate strains of nr. 9 mutually differed very strongly and the inhibiting effect on the whole was considerably weaker than that of the strains of *Penicillium* 18. Among the latter one strain (18a) was found, which even in the case of dilution up to 1/128 inhibited every growth of *Pythium*. Almost entirely with this strain the further investigations were carried on.

§ 2. *Influence of concentration of saccharose as carbon-source on the formation of growth inhibiting substances.*

Up till now the experiments had been made with Knop + 0,5% saccharose. This was no reason however to doubt that possibly an even stronger inhibiting effect could be obtained by making some alterations in the composition of the liquid medium. First an experiment was made with alterations in the quantity of the carbon-source. From table 8 appears, that this happens to be the most important substance. The great influence exercised by the concentration of saccharose makes it seem very probable, that the inhibiting substance is a metabolic product of the carbon-source.

TABLE 8.

Inhibiting effect of metabolic products of *Penicillium* 18 on growth of *Pythium de Baryanum*.

Period of growth of Penicillium culture	concentration of saccharose. 1%	2%	3%	4%	5%
	Dilution of metabolism fluid inhibiting growth of Pythium				
12 days	1/10	1/40	1/160	1/160	1/80
19 ,,	1/64	1/128	1/512	1/256	1/128
26 ,,	1/100	1/200	1/400	1/200	1/100
41 ,,	1/50	1/200	1/400	1/400	1/200

In the beginning the concentration of 3% saccharose has the

most inhibiting effect. Later on the effect of the 4% solution is just so strong, as shown by the table. In solutions stronger than those mentioned in the table a *total* inhibition of the growth no longer occurs; frequently however a strong *partial* inhibition may occur.

The strongest inhibition in this experiment was caused by the 4% concentration after 41 days. The vertical growth in the control-tubes for this series was 52 and 53 mm. In 1/800 dilution of the 4% this was 10 mm., in 1/1600 28 mm., in 1/3200 39 mm. For the 3% sacch. these numbers were respectively 20 mm, 41 mm, 48 mm. This shows a distinctly stronger effect of 4% saccharose than of the 3%. In the case of the 4% the inhibiting effect is still to be observed even in a dilution of 1 on 3200. The strongest toxicity, reacted as yet, was found in another series with Knop and 4% saccharose. In the tubes with the dilution 1/1280 no growth was yet to be found after 5 days. In the control-tubes the surface of the medium (55 mm.) was reached then. In the tube with dilution 1/5260 the inhibition was still noticeable.

A week after the inoculation a slight growth was too found in the tube with concentration 1/1280. After 3 weeks this was also the case in the tube with concentration 1/640, which shows, that the inhibiting capacity of the liquid medium gradually weakens in conservation. This had also been proved in a different way. A series of dilutions was inoculated directly after the sterilizing from the liquid medium of a *Penicillium* culture. A similar series was inoculated 14 days later. The inhibiting effect of the latter series was half so weak.

I have not been able to determine whether in the stronger concentrations of the inhibiting products *Pythium* is killed. This is however rather probable. In literature I have only been able to find 1 example of as strong a toxic effect as could be stated here for the *Penicillium*. Fleming (1929) discovered the strongly antagonistic reaction of a fungus versus some bacteria, pathogenic for human beings. This proved to be a *Penicillium*. Later on Clutterbuck, Lovel and Raystrick (1932) and Reid (1933, 1935) experimented with the same organism. The maximal dilution in which a total inhibition of growth still occurred, was at 1/1280, the same as with our *Penicillium*. In phytopathological literature I could

not find data about a similar strong formation of toxin as by the *Penicillium* of Fleming and by ours. The strongest inhibitive action mentioned, is that of the filtrate of *Diplodia Zeae* versus the fungus itself; after dilution up to 1/125 the growth was still quite inhibited. (Kent, 1938).

§ 3. *Influence of the nature of the carbon-source.*

It was proved by further investigations, that besides the concentration of the carbon-source, the nature of the latter is also of great influence on the formation of the inhibiting substances.

Penicillium was inoculated in tubes with different sterile media (agars, gelatin-media, plant-parts and nutritive solutions). After some days 10 cc of sterile water was added to each tube with solid medium. Of each medium the liquid from one tube was poured into another after 4 days; this was sterilized then in the ordinary way and after cooling down, inoculated with *Pythium de Baryanum*. In 6 of the tubes no growth occurred. The media of these tubes were: Sabouraud-agar, malt-agar, X-agar (= cherry, oatmeal, peptone, glucose, saccharose), Daucus-root, malt-salep-agar, and diluted cherry juice. Each of these 6 media contained mono- or di-saccharides or both together. Though *Penicillium* had developed well on the media with chiefly amylum as a carbon-source, in none of the concerning tubes a clearly antagonistic effect was to be found.

Eight days after the addition of water to the *Penicillium*-cultures an experiment was made again. Besides the 6 media, already mentioned, broth-pepton-glucose-saccharose was now among the media with the most strongly inhibiting effect.

The impression, that the nature of the carbon-source might prove to be an important factor, was strenghtened a good deal by this experiment. Subsequently a number of carbon-sources in synthetic liquid media were compared for that reason. Of some media 3, of the other 2 parallel-flasks, were inoculated again and again. In each flask of 100 cc contents 70 cc Knop + 2% carbon-source was sterilized and inoculated with *Penicillium*-spores. These were taken from a culture, the inhibiting capacity of which had been ascertained. It was tried to make the quantity and the nature

of the inoculation-material as equal as possible for the various flasks. In former experiments now and then unexpected differences in the inhibiting capacity had occurred. As a rule there had been an inoculation then with a drop of spore-suspension. *Penicillium*-conidia however can not but with great difficulty be equally divided in a liquid, and the amount of inoculationmaterial may have great influence on the development of a culture. In this case conidia were shaken off from the mother-culture in water and this was done so strongly as to keep the balls of conidia, held together by air, as small as possible. Then the liquid was poured into a Petri-dish, on which a rather equal layer of conidia was floating now. From this again and again a loopful was skimmed off and inoculated in a flask. In this way we succeeded in obtaining, that in by far the most cases a strong correspondence was to be found between the parallel-cultures of the same medium. Only in 2 of the media (galactose and glucose) a strong difference between the parallel-cultures had to be stated. The reason of this could not be ascertained. Our experiences in the tests made so

TABLE 9.

Inhibiting capacity of the metabolic products of *Penicillium expansum* with different carbon-sources.

maltose . . .	a	1 to 656	inuline . . .	a	1 to 95
	b	,, ,, 582		b	,, ,, 76
	c	,, ,, 600	amyl. sol. . .	a	,, ,, 59
sacch.	a	,, ,, 382	,,	b	,, ,, 97
	b	,, ,, 382	malt extr. . .	a	,, ,, 88
	c	,, ,, 370		b	,, ,, 86
laevulose . . .	a	,, ,, 378	manniet . . .	a	,, ,, 15
	b	,, ,, 386		b	,, ,, 32
	c	,, ,, 310	yeast	a	,, ,, 1,87
galact.	a	,, ,, 932		b	,, ,, 1,91
	b	,, ,, 127	lactose . . .	a	,, ,, 1,71
glucose	a	,, ,, 96		b	,, ,, 1,84
	b	,, ,, 364	glycocoll . .	a	,, ,, 1,50
dextrine	a	,, ,, 182		b	,, ,, 1,50
	b	,, ,, 176	asparagine . .		,, ,, 1.29

far, point to the possibility of great variability (saltations?) which concern the morphology as well as the physiology of the fungus. According to Clutterbuck a.o. (1932) such phenomena will also occur with the *Penicillium* of Fleming.

At different dates 10 cc. of the liquid medium was taken from each flask and tested in the ordinary way on its inhibiting capacity. The 4 weeks-old cultures showed the strongest effect. The figures in table 9 refer to the cultures of that age.

In this table figures are given which approximate the actual inhibiting capacity. The figures have been calculated through interpolation between the weakest concentration at which *total inhibition* still occurs, and the strongest concentration at which *growth* still occurs.

The mono- and di-saccharides (with the exception of lactose) are the best carbon-sources. The *inhibiting power* of the media with polysaccharides as a carbon-source was decidedly weaker; The *growth* however of *Penicillium* on the media with polysaccharides was rather stronger than slighter. The results of these experiments have proved that the inhibiting substances are metabolic products of carbon compounds.

§ 4. *Some characteristics of the metabolic products of Penicillium expansum.*

The stability of the inhibiting power was influenced by treatment of the cultures at 115° C (for one hour) and at 110° C (for 30 minutes), to such a degree that the inhibiting power was attenuated to 50%.

When the culture liquid was evaporated down to 10% of the volume and filled up to the original volume with uninoculated medium, the inhibiting power remained unchanged. The toxins are absorbed by kaolin and norit. Sometimes the pH was determined of the strongest dilution of metabolic substances that entirely inhibited the *Pythium* cultures. The pH proved to be between 5 and 6; this hydrogen-ion concentration is very well tolerated by *Pythium*. So it cannot have caused inhibition.

§ 5. *Experiments in pure cultures in tubes with lucerne and Pythium de Baryanum.*

Photo 9 illustrates the inhibitional power of the strong antagonistic liquid. The parasitism of *Pythium* was inhibited by 1/16 dilution. In this case the seedlings were decidedly damaged by the strongest concentrations of the toxines.

In a subsequent experiment the inhibiting power of the metabolic products against *Pythium* was before determined in a series of dilutions, with *Pythium* only. Down to 1/128 growth of *Pythium* was inhibited.

In the cultures with lucerne and *Pythium* the parasitism of *Pythium* was inhibited by 1/1 to 1/32 dilution. In the cultures with undiluted medium the seedlings were damaged. Dilutions from 1/10 on were harmless.

In a third experiment the inhibiting effect of cultures of different age were compared.

TABLE 10.

Inhibiting effect of sterilized liquid medium of *Penicillium* cultures of different age.

	5 days old Penicilliumcult.		15 days old Penicilliumcult.	
Control	4	0		
,,	5	0		
Pythium de Bar.	0	1		
,, ,,	0	2		
,, ,, ,, with liquid medium 1/1	0	5	0	4
,, ,, ,, ,, ,, ,, 1/2	4	0	0	4
,, ,, ,, ,, ,, ,, 1/4	5	0	0	0
,, ,, ,, ,, ,, ,, 1/8	4	0	5	0
,, ,, ,, ,, ,, ,, 1/16	5	0	5	0
,, ,, ,, ,, ,, ,, 1/32	0	5	5	0
,, ,, ,, ,, ,, ,, 1/64	0	5	5	5
Number of seedlings:	healthy	diseased or dead	healthy	diseased or dead

Lucerne in tubes with Knop and filterpaper 5 seeds pro tube.

The influence of the age of the *Penicillium* culture is clear; in the liquid of the 5 days old culture down to 1/16, *Pythium* was totally inhibited. With the 15 days old cultures down to 1/32 the same result has been obtained. In the undiluted liquid of the 5 days old culture the seedlings were badly damaged by the toxines. With the 15 days old cultures, with stronger inhibiting capacity, the dilution down to 1/2 had this effect.

§ 6. *Experiments in pots with sterilized gardensoil.*

A comparison was made between influence of liquid sterilized medium and living *Penicillium*-conidia only. (photo 10).

The sterilized liquid was applied in three concentrations: 50 cc., 12,5 cc and 3 cc. (in pots with 200 cc soil) in the two latter cases, filled up to 50 cc with water. (4 pots with 40 seeds each in each series).

Controls healthy; pots with *Pythium:* seedlings diseased and dead, pro pot maximum 5 seedlings developed but very poorly.

The sterilized liquid, when added, inhibited parasitism entirely in the cases of 50 cc and 12,5 cc pro pot. With 3 cc no result.

The addition of living *Penicillium* conidia however did not influence the attack by *Pythium;* neither the conidia with the metabolic products nor without them.

The explanation may be the following:

It is probable that in certain series the chemical substances, necessary for the formation of inhibiting substances are not present in the soil, or not in sufficient quantities. The substances should then be added with the antagonists themselves. This of course can be done by applying with the organism its culture medium.

In this connection I want to refer to the considerations of Th. van Eek (1937) in his chapter on „Analysis of some parasitic characters".

§ 7. *Treatment of gardensoil with cultures of Penicillium expansum.*

Lucerne was sown in gardensoil in pots (175 cc) in the hothouse. Half of these pots obtained 50 cc of a living *Penicillium*

culture in Knop-saccharose, the controls an equal quantity of water only. This gardensoil contained virulent parasites, so that after ten days 67% of the seedlings in the controls were dead, whereas this figure was reduced to 12%, when *Penicillium* had been added (photo 11).

Penicillium proved to be antagonistic to the present parasites, probably *Pythium* species.

Another experiment was made with rye-kernels infected with *Fusarium* spec. Part of these seeds were immersed for 3 or 5 hours in sterilized liquid medium of *Penicillium*, the controls in water only (temperatures 15—20° and 28°). After this treatment the kernels were laid out in Petri-dishes with oat-meal-agar (25 seeds each).

The *Fusarium* was very distinctly inhibited in its development, but this inhibition only lasted for a short time. After 4 days it was checked. Nevertheless the inhibitory power of *Penicillium* seems not to be restricted to *Pythium*.

A comparison was made between the toxic influence of the *Penicillium* liquid medium and a solution of corrosive sublimate. A series of dilutions of an 1/10% solution was prepared. The result was that the strongest dilutions which inhibited the growth of *Pythium* was 1/640, in one case the liquid medium of *Penicillium* producing the same inhibition of growth has been diluted down to 1/1280.

We must thus conclude that the toxicity of the *Penicillium* liquid medium equals that of 1/5% of corrosive sublimate.

§ 8. *Agreement and difference with the penicillin of Fleming.*

The *Penicillium* investigated by Fleming belongs to the *chrysogenum* group *(P. notatum?)*.

This organism is very variable, as regards the morphological and the physiological characters (Clutterbuck a. o.). These authors in their experiments used a solution containing 4% glucose; a dilution down to 1/1280 inhibited the growth of *Streptococcus pneumoniae*.

The same critical dilution was observed for our *Penicillium expansum*, but with 4% saccharose in the medium. Both organisms

form metabolic substances in the liquid media which can be stored for quite long periods, without great loss of inhibiting power. Flemings *Penicillium* wants: „adjusting to pH 5 and 6", whilst ours is not adapted so strictly. There is however a distinct difference between the pH of the metabolic products. After Fleming the pH of the metabolic products is 9; these of our *Penicillium* are acid.

CHAPTER V

DISCUSSION ON POSSIBILITIES OF PRACTICAL APPLICATION

Where we find an attacking of higher plants by micro-organisms, the formation of toxin by the latter will frequently play an important part. The degree in which this toxin-formation occurs, will be strongly influenced again by environmental factors. Important factors in this case certainly are: temperature, moisture, pH and in particular the presence and the amount of certain nitrogen- and carbon-sources and other substances, necessary for the forming of these toxins. When making inoculation-experiments with plant-parasites it will therefore be of vital importance whether we only apply the parasite or at the same time the medium on which it has been grown. When in the soil certain substances, necessary for the formation of toxin by the parasite, are lacking or are only scantily present, the inoculation may be a failure; it can however be successful even in this case, when, besides spores or mycelium of the parasite, the substances, needed for the toxin-formation, are also given.

The age of the culture with which inoculations are made, is also of the utmost importance. In older cultures the necessary substances may already have been consumed and besides, the toxic substances formed may have been transmuted again. The failure of inoculation-experiments with older cultures of plant-parasites can probably be explained in this way in many cases. In other cases the presence of staling-products again may be of some influence: that is to say, of products, which are just as detrimental or even more so for the parasite itself as for the host-plant. For the rest it will be better to be very careful when drawing conclusions from those inoculation-experiments, in which besides the parasite also

substances are introduced into the soil, which under normal conditions, do not occur or at least not in that degree.

What has been said here about plant-parasites, also holds good for antagonistic organisms; both names in fact often are applied to one and the same microorganism. In artificial experiments the result may often be obtained, that by the addition of a second microorganism the parasitic activity of another may be quite or nearly quite inhibited. However this does not yet imply, that under conditions, occurring in practice, we only have to introduce the antagonist into the soil to prevent the occurrence of the parasite. Even in the case of the antagonistic reaction of one microorganism on the other, the formation of inhibiting substances will generally be an important factor and all this includes that here too the above-mentioned environmental factors may have a considerable influence.

So it will be of special importance whether only the fungus or bacterium is put into the soil or whether by the side of it the medium together with the toxins already formed and the substances by which the latter can be formed, is added as well.

The combatting of plant diseases through antagonistic organisms thus will often only be possible when making some favorable changes in the environmental factors, besides of the applying of the favorable organisms. In my opinion the former will as a rule be of greater importance than the adding of the organisms.

The application of filtered or sterilized cultures will in my opinion offer the best opportunity for a practical useful application. It has been proved that the toxins can be formed in very strong concentrations in the culture-liquids and these concentrations can even be intensified a good many times by evaporation. There is also the possibility of the isolation of the inhibiting substances.

This reveals to me the possibility, that soil disinfection might take place with quantities, that can practically be applied. When treated in this way, the application of the antagonism for the combatting of plant diseases really implies that the already rather large number of fungicides is increased. However many fungicides of mineral origin are not always obtainable, or with much difficulty under certain circumstances and it is then that the sanitary conditions of agricultural and horticultural plants are of vital interest.

In many cases good results may be obtained with various organic disinfectants as formalin, phenol, acetic acid, etc. For this however a treatment is required, which takes place a long time before the sowing of the plant. The disinfectants must first have altogether disappeared, as they might do harm to the seedlings. When applying the metabolic products of *Penicillium expansum*, it has turned out however, that these substances may be applied a few days before the sowing takes place to artificially inoculated soil as well as to diseased, non-sterilized soil and no damage was found to have been done to lucerne, sown in that soil. Such was the case too with concentrations of these substances, that were sufficient to inhibit a powerful attack by root-parasites. It may be expected, that besides for treating the soil, metabolic products will also prove useful for disinfection of the seed and for spraying of the parts of the plant above the soil.

Probably more extensive investigations will bring to light that more micro-organisms are capable of forming inhibiting substances, just as strong or even stronger than the *Penicillium* of Fleming or *Penicillium expansum*. It is also to be expected, that besides mono- and di-saccharids, all sorts of other organic carbon-combinations will prove practical for use. In this connection we may mention in the first place all sorts of products of refuse from industries. Besides fungicides and bactericides we will probably be able to isolate all kinds of other valuable substances from these products of refuse: a vast field of work for microbiologists and for the industries concerned.

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Control — Pythium mamillatum — Pythium irregulare — Pythium volutum — Pythium de Baryanum

Photo 1. Parasitism of different Pythiumspecies versus Agrostis stolonifera

sterile soil — non sterile soil

Photo 2. Inoculation with Pythium volutum in sterile and non sterile soil. Antagonism of soil organisms versus Pythium volutum.

Control — P. volutum — P. mamillatum — P. volutum + P. mamillatum

Photo 3. Antagonism of Pythium mamillatum versus Pythium volutum.

3 tubes Pythium with Pullularia filtrate
1 tube Pythium with Pullularia culture

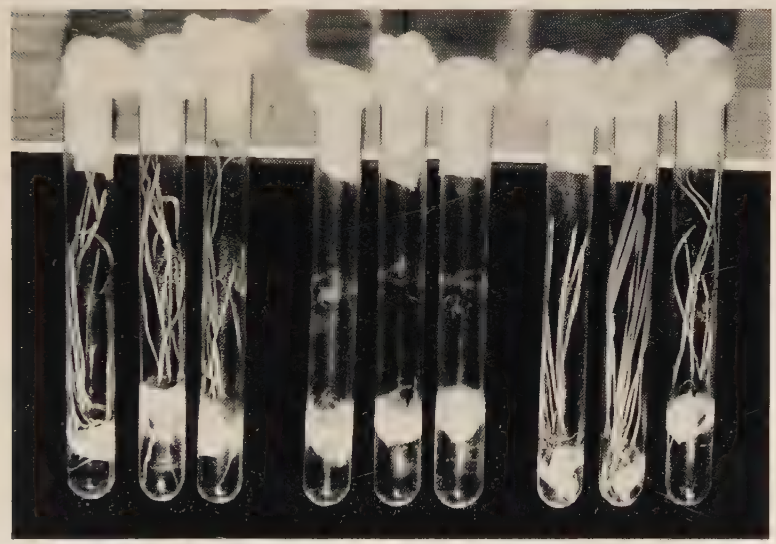
Pythium with sterilized and not filtered Pull. culture

Pythium de Baryanum control

Photo 4. Inhibiting effect of filtrate of Pullularia pullulans versus Pythium de Baryanum.
No inhibiting effect of sterilized and not filtered cultures of Pullularia pullulans.

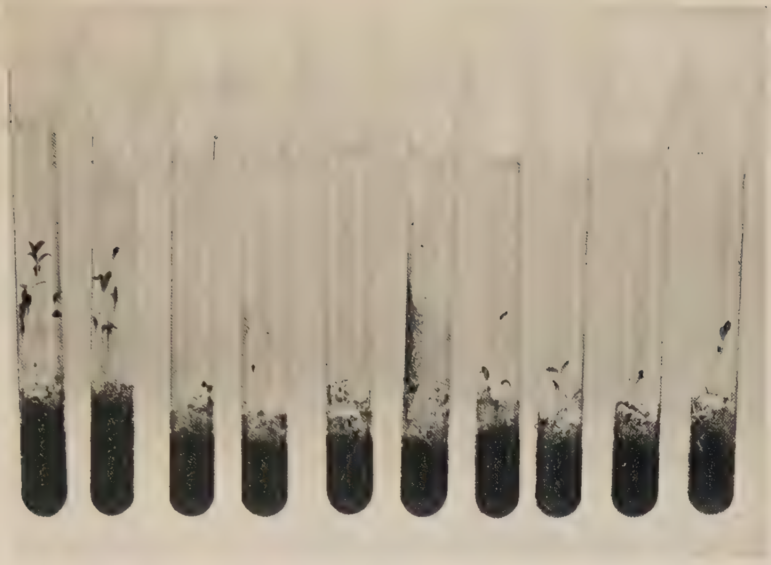
Pythium de Bar. and Pull. pullulans | Pythium de Bar. | Control.

Photo 5. Antagonism of Pullularia pullulans versus Pythium de Baryanum. (Development of roots).



no infection | P. de Baryanum | Pythium de Bar. with Pull. filtrate

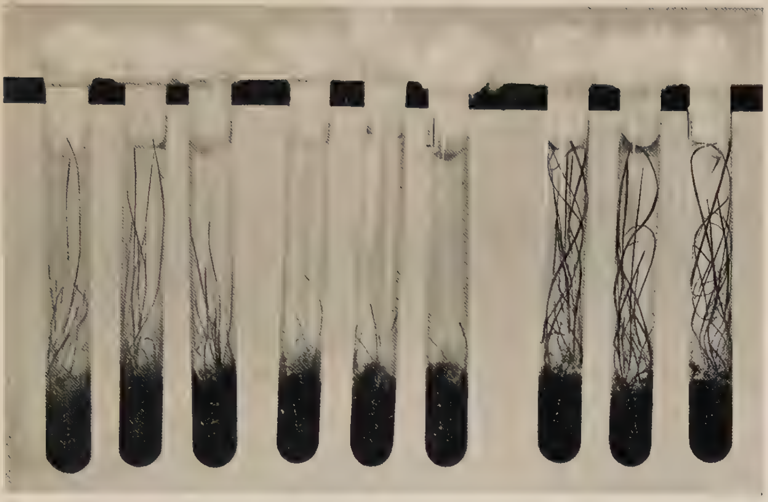
Photo 6. Inhibiting effect of filtrate of Pullularia pullulans on parasitism of Pythium de Baryanum on Lolium.



Control | Pythium | Pythium + Bact. 5 | Pythium + Bact. 7 | Pythium + Sporotrichum spec.

Photo 7. Antagonism of selected soil organisms versus Pythium de Baryanum on lucerne. Antagonists separately and in combinations.

Pythium + Verticill. spec | Pythium + Vertic. + Bact. 5 | Pythium + Vertic. + Bact. 7 | Pythium + Sporotr. + Bact. 7



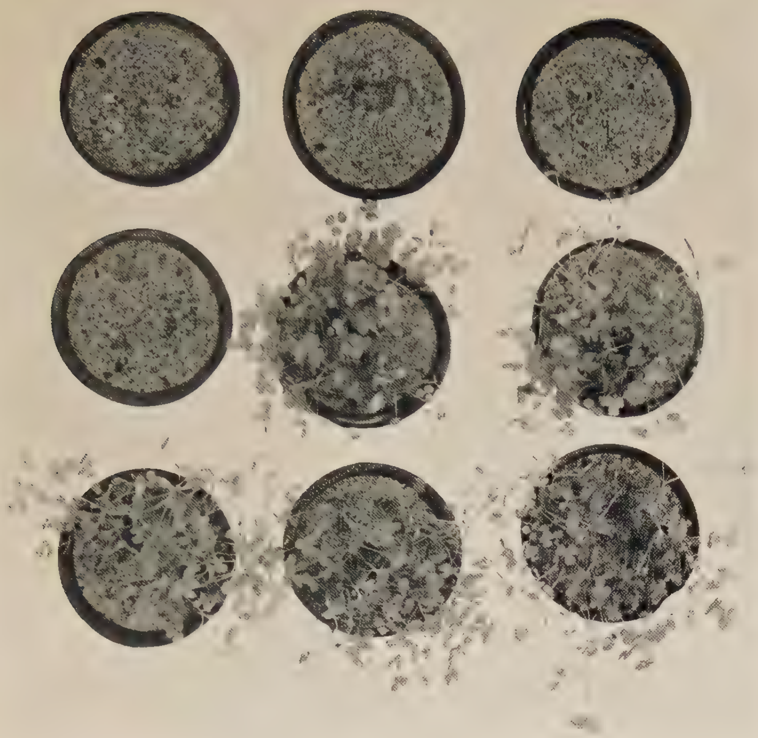
Control Pythium volutum Pythium volutum with drop of soil suspension

Photo 8. Antagonism of soil organisms against parasitism of Pythium volutum on Agrostis. Drop of soil suspension.

$^1/_2$ $^1/_4$ $^1/_8$ $^1/_{16}$ $^1/_{32}$

Control Pythium Pythium and liquid medium

Photo 9. Inhibiting effect of sterilized liquid medium of Penicillium expansum versus Pythium de Baryanum on lucerne (diluted up to $^1/_{16}$ effective, $^1/_{32}$ no effect.)



Pyth. + Penic. conidia	Pyth. + 3 cc Penic. liquid	Pyth. + Penic. conidia + 3 cc Penicill. liquid
Pythium	12,5 cc	12,5 cc
no infection	50 cc	50 cc

Photo 10. Antagonism of Penicillium expansum against Pythium on lucerne in pots. Sterile soil.

Attack of soil organisms — Inhibiting effect of Penicillium expansum

Photo 11. Lucerne in non sterile garden soil in pots. Attack by soil organisms. Inhibiting effect on the attack by addition of living culture of Penicillium expansum.